Studies on Some Immune Properties of the Pancreatic Progenitor Cells Derived from Human Fetal Pancreas

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ABSTRACT

The potential use of human fetal pancreas as an alternative source for transplantable beta-cells to treating type 1 diabetes mellitus depends on its immune privilege as well as high capacity to proliferate and differentiate. This notion has been substantiated by a recent study showing a reduced immunogenicity of first-trimester human fetal pancreatic tissue. Meanwhile, we have successfully isolated, characterized and cultured a population of pancreatic progenitor cells (PPCs) derived from human fetal pancreas that is amenable to growth and differentiation into insulin-producing islet-like cell clusters (ICCs). Despite this, the immunogenicity of these PPCs and ICCs has yet to be characterized. We thus hypothesize that the PPCs and ICCs derived from first-trimester possess lower immunogenicity than that derived from second-trimester of human fetal pancreas; we tested this hypothesis by characterizing and comparing the immune properties of these cells.

In order to achieve this, we first investigated the expression profile of selected immune-related genes by real-time PCR. Expression of the major histocompatibility complex class I (MHC-I) molecules upon stimulation of interferon-gamma (IFN-γ) was further examined by flow cytometry. Results showed positive gene expression of several

selected immune-related genes including MHC molecules, complement components (C3), apoptotic markers (TNFSF10), chemokine ligands (CCL19), innate molecules (B7H4). Both co-stimulatory molecule, CD80 and CD86, were found to be negative in both PPCs and ICCs. Upon stimulation of IFN-γ, MHC-I expression on PPCs was upregulated in a dose-dependent manner. Comparison of these immunological markers in PPCs from the first and second trimesters was also undertaken. A differential gene expression of MHC-I was observed in PPCs from 9 to 14 weeks of gestation, with an enhanced expression from the second trimester. Expression of the pro-inflammatory genes was significantly elevated in PPCs from the second trimester, indicating an immuno-privilege of PPCs from the first trimester. We also confirmed a higher inducibility of MHC-I and MHC-II expression in PPCs from the second-trimester by IFN-γ.

Seondly, we investigated the alloreactivity of the progenitors *in vitro* by mixed lymphocyte reaction (MLR) and the proliferation of human lymphocytes were determined by BrdU incorporation. Results revealed a significant higher stimulation of lymphocytes in MLR of PPCs from the second trimester. A higher production of IFN-γ was also observed in Phytohemagglutinin (PHA-P) stimulated MLR with second trimester. Lastly, we further investigated the *in vivo* xenoreactivity by transplantation of ICCs into a mouse

model of diabetes. Although both first- and second-trimester ICC grafts failed to normalize blood glucose in the diabetic mice, immunohistochemistry analysis of grafted-kidney revealed a significant increase in lymphocyte infiltration on second-trimester islet grafts, implicating a reduced immunogenicity in the ICCs of first over second trimester.

Taken all these data together, we conclude that PPCs of first-trimester has an immune privilege over second-trimester derived from human fetal pancreas. The present study provides not only insights into the immunological properties of PPCs and ICCs but also perspective into the use of first-trimester PPCs as an alternative source for transplantable islets with reduced cellular infiltration.

論文摘要

基於其擁有免疫豁免以及較高細胞增殖及分化能力的特性，人類胚胎胰臟組織有潛力代替可移植胰腺細胞以治療一型糖尿病。相關報告也提到了由妊娠早期收取的人類胚胎胰臟組織中的免疫原性較妊娠中期所收取的爲低。本研究小組在早前已就關於人類胚胎胰腺祖細胞（Pancreatic Progenitor Cells）的分離、培養以及表征發表了文獻，這些胰腺祖細胞更可以增殖及分化成可分泌胰島素的"類小島細胞球"（ICCs）。由於目前我們還未對這些細胞的免疫原性作出研究，本報告旨在探究人類胚胎胰腺祖細胞及類小島細胞球的免疫原性，並證明由妊娠早期收取的祖細胞比妊娠中期更具免疫豁免的特性。

我們先以實時螢光定量聚合酶連鎖反應（real-time PCR）來測試一些與免疫系統有關的訊息核糖核酸（mRNA）的表達，同時使用流式細胞儀技術測試干擾素-γ（IFN-γ）對細胞上相容性綜合複合體（MHC）表達的影響。結果證明相容性綜合複合體、補體 3（C3）、腫瘤壞死因子受體超家族 10 （TNFSF10）、趨化因子配體 19 （CCL19）、B7 至 H4(B7H4) 訊息核糖核酸在胚胎胰腺祖細胞及類小島細胞球上的表達。相反，這些細胞上並未表達共刺激分子 CD80 和 CD86 的訊息核糖核酸。此外，干擾素-γ 對祖細胞上 MHC-I 的表達有劑量依賴性誘發的效果。就此，我們進一步比較由妊娠早期與妊娠中期收取的人類胚胎胰腺祖細胞中的免疫原性。結果發現 MHC-I 的表達在

第九至第十四週胰腺祖細胞有差異，當中以妊娠中期祖細胞的表達較高。研究亦發現一些促炎因子在妊娠中期的祖細胞也有較高表達。此外，相比於妊娠早期的祖細胞，干擾素-γ 可在妊娠中期細胞誘發更多 MHC-I 及 MHC-II。

爲進一步探究這些祖細胞在培養環境中的同種異體反應，我們進行了胰腺祖細胞的同種異體混合淋巴細胞反應（Mixed lymphocyte reaction），並以 5 -溴脫氧尿嘧啶核苷（BrdU）標記來定量淋巴細胞的細胞增殖。結果發現妊娠中期的祖細胞可誘發更高淋巴細胞的細胞增殖。當植物血球凝集素(PHA-P)加進混合淋巴細胞反應內，妊娠中期比妊娠早期的祖細胞可使淋巴細胞產生更多干擾素-γ. 最後，我們把類小島細胞球移植到患有糖尿病的小鼠中以評估它的異種異體反應。雖然兩組的移植體都未能使小鼠的血糖回復正常水平，免疫組織化學分析反映妊娠中期的類小島細胞球在腎囊中的移植體有較高的淋巴細胞浸潤程度，進一步證明妊娠早期的類小島細胞球有較低的免疫原性。

總括而言，結果顯示從妊娠早期收取的人類胚胎胰腺祖細胞比與妊娠中期有更高的免疫豁免。因此，本報告不但描述了人類胚胎胰腺祖細胞以類小島細胞球免疫原性的表征，更預料妊娠早期祖細胞有更高的潛力有作爲胰腺移植細胞。

LIST OF PUBLICATIONS & ACADEMIC ACTIVITIES

ORIGINAL ARTICLES

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TABLE OF CONTENTS

Abstract..... I

List of Publications..... VI

Acknowledgements..... VIII

Table of Contents..... X

List of Figures..... XV

List of Tables..... XVIII

List of Abbreviations..... XIX

CHAPTER 1
INTRODUCTION

1.1 The Pancreas..... 2
1.1.1 Structure of pancreas..... 2
1.1.2 Structure and function of exocrine pancreas..... 6
1.1.3 Structure and function of endocrine pancreas..... 9
1.1.3.1 Pancreatic islet and islet cells..... 9
1.1.3.2 Glucose-stimulated insulin secretion from islets..... 12

1.2 Type 1 Diabetes Mellitus (T1DM)..... 14
1.2.1 Pathophysiology of Diabetes Mellitus..... 14
1.2.2 Autoimmunity in T1DM..... 17
1.2.3 Management of T1DM..... 20
1.2.3.1 Insulin replacement..... 20
1.2.3.2 Pancreas and islet transplantation..... 21
1.2.3.3 Stem-cell-based transplantation..... 22

1.3 The Adaptive Immune System..... 26
1.3.1 T-lymphocytes..... 26
1.3.2 B-lymphocytes..... 29

1.3.3 Major histocompatibility complex (MHC)..... 30
1.3.3.1 Classification of MHC molecules..... 30
1.3.3.2 Structure of MHC class I and II molecules..... 32
1.3.3.3 Function and regulation of MHC molecules..... 34
1.3.4 HLA-G and its immuno-modulatory properties..... 36

1.4 Transplantation Rejection..... 40
1.4.1 Mechanisms involved in transplantation rejection..... 40
1.4.2 Immunobiology of rejection..... 41
1.4.2.1 Direct allorecognition pathway..... 42
1.4.2.2 Indirect allorecognition pathway..... 43
1.4.2.3 Semi-direct allorecognition pathway..... 43
1.4.3 Xenotransplantation..... 46

1.5 Cytokines and Immunity..... 48
1.5.1 Interferons..... 48
1.5.1.1 Interferon-γ and its immune regulation..... 49
1.5.1.2 Effect and kinetics of interferon-γ on MHC molecules expression..... 53
1.5.1.3 Regulation of interferon-γ production..... 56
1.5.2 Interlukins..... 58
1.5.2.1 IL-10 and its immune regulation..... 58
1.5.2.2 IL-10 and HLA-G..... 59

1.6 Stem Cells and their Immunogenicity..... 62
1.6.1 Embroynic stem cells..... 62
1.6.2 Mesenchymal stem cells..... 64
1.6.3 Neural stem cells..... 68
1.6.4 Fetal stem cells..... 69
1.6.5 Potential immuno-study in human fetal pancreatic stem cells..... 70

1.7 Aims and Objectives of study..... 72

CHAPTER 2
MATERIALS AND METHODS

2.1 Isolation of Pancreatic Progenitors (PPCs) from Human Fetal Pancreas and Induction of Islet-like Cell Cluster (ICCs) Differentiation..... 75
2.1.1 Tissue procurement..... 75
2.1.2 Tissue processing and PPCs culture..... 75
2.1.3 In vitro differentiation of PPCs into ICCs..... 78
2.1.4 Interferon-γ and IL-10 treatment..... 80

2.2 Cell culture of human placental Choriocarcinoma JEG-3 Cell Line..... 81

2.3 RNA Expression Detection..... 82
2.3.1 RNA isolation..... 82
2.3.2 Reverse transcriptase (RT) 83
2.3.3 Design of primers for Polymerase Chain Reaction (PCR) and Real-time PCR 84
2.3.4 PCR..... 86
2.3.5 Real-time PCR analysis..... 88
2.3.6 Calculation using the comparative C_T method..... 90

2.4 Flow Cytometry..... 91

2.5 Western Blotting Analysis..... 93
2.5.1 Protein extraction and quantification..... 93
2.5.2 Western blotting..... 93

2.6 Mixed Lymphocyte Reaction (MLR) 95
2.6.1 Isolation of peripheral blood mononuclear cells (PBMCs) 95
2.6.2 PPC-PBMCs MLR..... 98
2.6.3 ICC-PBMCs MLR..... 98
2.6.4 Proliferation assay..... 99

2.7 ICC Transplantation..... 101
2.7.1 Streptozotocin-induced diabetic animals for transplantation..... 101
2.7.2 Procedures of ICCs transplantation..... 102

2.8 Histological Analysis of ICC Graft..... 105
2.8.1 H&E staining..... 105
2.8.2 DAB staining..... 106
2.8.3 Immunofluorescence staining..... 107

2.9 Enzyme-linked Immunosorbent Assay (ELISA) 109

2.10 Statistical Data Analysis..... 110

CHAPTER 3
RESULTS

3.1 Immuno-characterization of PPCs and ICCs..... 112

3.2 Effect of cytokines on immune-properties of PPCs and ICCs..... 115
3.2.1 Effect of IFN-γ on MHC-I expression in PPCs..... 115
3.2.2 Effect of IFN-γ and IL-10 on HLA-G expression in PPCs and ICCs..... 119
3.2.3 Effect of IFN-γ on B7H4 expression in PPCs..... 123

3.3 Comparison of immune-properties of PPCs and ICCs from 1st and 2nd trimester..... 125
3.3.1 Differential expression of MHC molecules in PPCs..... 125
3.3.2 Different immune-related gene expression in PPCs and ICCs..... 128
3.3.3 Comparison of IFN-γ activated MHC molecules expression in PPCs/ICCs..... 134
3.3.4 Comparison of other IFN-γ activated genes expression in PPCs..... 139

3.4 Mixed lymphocyte reaction of PPCs from 1st and 2nd trimester..... 143
3.4.1 Effect of PPCs on proliferation of PBMC..... 143
3.4.2 Effect of ICCs on proliferation of PBMC..... 145
3.4.3 Effect of PPCs on cytokine production in PBMC..... 149

3.5 Xenotransplantation of ICCs into diabetic mouse model..... 152
3.5.1 Blood glucose level of diabetic mice after transplantation..... 152
3.5.2 Histological evaluation of transplanted ICCs grafts..... 154
3.5.3 Infiltration of CD45 into transplanted grafts of 1st and 2nd trimester..... 158

CHAPTER 4
DISCUSSION

4.1 Expression of selected immuno-regulated genes in PPCs and ICCs..... 163
4.2 Effect of IFN-g and IL-10 on expression of immuno-regulated genes in PPCs and ICCs..... 166
4.3 In vitro studies on immunogenicity of PPCs and ICCs from first and second trimester..... 171
4.3.1 Immune-related genes expression..... 171
4.3.2 IFN-γ activated gene expression..... 173
4.3.3 Mixed lymphocyte reaction..... 175
4.3.4 Cytokine production of PBMC in MLR..... 179
4.4 In vivo Xenotransplantation of ICCs into diabetic mouse model..... 181
4.5 Conclusion..... 187
4.6 Further studies..... 188

CHAPTER 5
BIBLIOGRAPHY

Bibliography by Alphabetical Order..... 189

LIST OF FIGURES

Figure 1.1	The macroscopic view of pancreas.....	3
Figure 1.2	The microscopic view of pancreas, pancreatic duct, acinar and islet of Langerhan.	5
Figure 1.3	Schematic diagram of glucose-stimulated insulin secretion (GSIS).....	13
Figure 1.4	Etiology of T1DM.....	19
Figure 1.5	Generation of insulin-expressing cells from pluripotent cells and organ-specific progenitor cells.	25
Figure 1.6	Costimulation in T-cells activation.....	28
Figure 1.7	Structure of MHC class I and II molecules.....	33
Figure 1.8	Primary transcript and isoforms of HLA-G.....	38
Figure 1.9	Direct and indirect T-cell recognition pathways.	45
Figure 1.10	MHC I and II induction through IFN-γ signal transduction pathway...	55
Figure 1.11	Classical and "jump start" model of production of IFN-γ by immune cells.....	57
Figure 1.12	Immunomodulatory effects of MSCs.....	67
Figure 2.1	Representative microscopic images showing the in vitro PPCs induction and ICCs differentiation.	79
Figure 2.2	The principle of SYBR Green detection in real-time PCR.....	89
Figure 2.3	The basic principle of flow cytometry and two classical data output formats.....	92
Figure 2.4	Schematic diagram of human peripheral blood mononuclear cells (PBMCs) isolation by ficoll gradient.....	97
Figure 2.5	The ICC transplantation procedures on C57BL/6J mice.....	104
Figure 3.1	The mRNA expression of some selected immune-related genes from PPCs and ICCs.....	113
Figure 3.2	Real-time RT-PCR analysis of the mRNA expression of MHC-I molecules from PPCs and human fetal pancreas.....	114
Figure 3.3	Real-time RT-PCR analysis of the mRNA expression of MHC-I molecules in IFN-γ treated PPCs.....	117
Figure 3.4	Flow cytometry analysis of surface MHC-I expression in IFN- γ untreated and treated PPCs.....	118
Figure 3.5	Real-time RT-PCR analysis of the mRNA expression of HLA-G on	

	IL-10 treated PPCs.....	120
Figure 3.6	Real-time RT-PCR analysis of the mRNA expression of HLA-G on IL-10 treated ICCs.....	121
Figure 3.7	Western blotting analysis of the HLA-G expression in JEG-3 and PPCs upon pre-treatment with IFN-γ and IL-10.....	122
Figure 3.8	RT-PCR analysis of the mRNA expression of B7H4 on IFN-γ treated PPCs.....	124
Figure 3.9	Real-time RT-PCR analysis of the mRNA expression of MHC-I in 9th-14th week PPCs.....	126
Figure 3.10	Real-time RT-PCR analysis of the mRNA expression of MHC-II in 9th-14th week PPCs.....	127
Figure 3.11	Real-time RT-PCR analysis of the mRNA expression of several pro-inflammatory molecules in PPCs of first and early second trimester.....	130
Figure 3.12	Real-time RT-PCR analysis of the mRNA expression of several immune-suppressing molecules in PPCs of first and early second trimester.....	131
Figure 3.13	Real-time RT-PCR analysis of the mRNA expression of several pro-inflammatory molecules in ICCs of first and early second trimester.....	132
Figure 3.14	Real-time RT-PCR analysis of the mRNA expression of several immune-suppressing molecules in ICCs of first and early second trimester.....	133
Figure 3.15	Real-time RT-PCR analysis of the mRNA expression of IFN-γ induced MHC-I in PPCs of first and second trimester.....	135
Figure 3.16	Flow cytometry analysis of surface MHC-I expression on IFN-γ treated PPCs.....	136
Figure 3.17	Real-time RT-PCR analysis of the mRNA expression of IFN-γ induced MHC-I in ICCs of first and second trimester.....	137
Figure 3.18	Flow cytometry analysis of surface MHC-I expression on IFN-γ treated ICCs.	138
Figure 3.19	Real-time RT-PCR analysis of the mRNA expression of IFN-γ induced MHC-II in PPCs of first and second trimester.....	140
Figure 3.20	Real-time RT-PCR analysis of the mRNA expression of IFN-γ induced HLA-G in PPCs of first and second trimester.....	141
Figure 3.21	Real-time RT-PCR analysis of the mRNA expression of IFN-γ	

	induced B7H4 in PPCs of first and second trimester.....	142
Figure 3.22	Brdu incorporation analysis of mixed lymphocyte reaction of human isolated PBMCs and PPCs.....	146
Figure 3.23	Brdu incorporation analysis of mixed lymphocyte reaction of human isolated PBMCs and PPCs.....	147
Figure 3.24	Brdu incorporation analysis of mixed lymphocyte reaction of human isolated PBMCs and a 13 week ICCs sample.....	148
Figure 3.25	IFN-γ cytokine ELISA analysis of medium collected from MLR of PHA-P stimulated PBMCs and PPCs.....	150
Figure 3.26	IL-10 cytokine ELISA analysis of medium collected from MLR of PHA-P stimulated-PBMCs and PPCs.....	151
Figure 3.27	Blood glucose level of STZ-induced diabetic C57/BL6 mice after transplanted with ICCs of first and second trimester.....	153
Figure 3.28	Histological analysis human fetal ICCs graft (T2/T4).....	155
Figure 3.29	Histological analysis human fetal ICCs graft (T7/T12).....	156
Figure 3.30	Immunohistochemical localization of human PDX-1 in the human fetal ICCs grafts.....	157
Figure 3.31	Histological analysis of first- and second-trimester human fetal ICCs grafts.	159

LIST OF TABLES

Table 1.1	Diagnosis of pre-diabetes and diabetes by Fasting Plasma Glucose (FPG) and Oral Glucose Tolerance Test (OGTT).....	16
Table 1.2	The inhibitory effect of HLA-G on various kinds of cells.....	39
Table 1.3	Some highlighted genes or proteins regulated by IFN-γ	52
Table 2.1	Primer-pairs used for the study of immuno-related gene expression in PPCs and ICCs.....	85
Table 2.2	Primer-pairs used for the study of immuno-related gene expression in PPCs and ICCs.....	87

LIST OF ABBREVIATIONS

ACh	**Acetylcholine**
Ang II	**Angiotensin II**
APC	**Antigen presenting cells**
ATP	**Adenosine triphosphate**
BrdU	**5-bromo-2-deoxyuridine**
C3	**Complement component 3**
CCK	**Cholecystokinin**
CCL19	**Chemokine (C-C motif) ligand 19**
CD45	**Cluster of Differentiation 45**
CD80	**Cluster of Differentiation 80**
CD86	**Cluster of Differentiation 86**
CSIF	**Cytokine synthesis inhibitory factor**
DAB	**3,3-diaminobenzidine**
DCs	**Dendritic cells**
E2	**Prostaglandin**
EBs	**Embryoid bodies**
EGF	**Epidermal growth factor**
ESC	**Embryonic stem cells**
Ex-4	**Exendin-4**
FPG	**Fasting plasma glucose**
GAD65	**Glutamic acid decarboxylase**
GAS	**IFN-gamma-activated sites**
GDM	**Gestational diabetes melitus**
GIP	**Gastric inhibitory peptide**
GLP-1	**Glucagon-like-peptides-1**
GSIS	**Glucose-stimulated insulin secretion**
HCO_3^-	**Sodium bicarbonate**
HGF	**Hepatic growth factor**
HLA	**Human leukocyte antigen**
HNF-4α	**Hepatocyte nuclear factor 4α**
HSCs	**Haemopoietic stem cells**
IA-2	**protein tyrosine phosphatase-related islet antigen 2**
ICCs	**Islet-like cell clusters**
IFN-γ	**Interferon-gamma**
IGT	**Impaired glucose tolerance**

IL-10	Interleukin-10
IL-12	Interleukin-12
ILTs	Immunoglobulin-like receptors
IPF-1	insulin promoter factor-1
IRF-1	Interferon regulatory factory-1
ISGs	Interferon-stimulated genes
ISRE	IFN-stimulated response elements
JAK	Janus kinase
LCA	Leukocyte common antigen
mHC	Minor histocompatibility complex
MHC-I	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MODY	Maturity onset diabetes of the young
MSC	Mesenchymal stem cells
NOD mice	non-obese diabetic mice
NPCs	Neural precursor cells
OGTT	Oral glucose tolerance test
PBMCs	Peripheral blood mononuclear cells
PDX-1	Pancreatic and duodenal homeobox 1
PHA-P	Phytohemagglutinin
PLC	Phospholipase C
PPCs	Pancreatic progenitor cells
STAT-1	Signal transducers and activators of transcription-1
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCR	T cell receptors
TGF-β	Transforming growth factor-β
Th1	T helper 1 cells
Th2	T helper 2 cells
TNF	Tumour necrosis factors
TNFSF	Tumor necrosis factor superfamily
Treg	T regulatory cells
VIP	Vasoactive intestinal polypeptide

CHAPTER 1

INTRODUCTION

1.1 The pancreas

1.1.1 Structure of pancreas

The pancreas is an organ composed of two structurally distinct but functionally integrated parts, i.e. endocrine and exocrine glands. Embryologically, they are derived from an outgrowth of primitive gut tube of definitive endoderm. The major functions of the endocrine and exocrine glands are to produce islet cell hormones (e.g. insulin and glucagon) and acinar cell digestive enzymes, along with ductal cell sodium bicarbonate, to maintain nutrient homeostasis and assimilation, respectively. Structurally, the pancreas is a flat and elongated organ, which is more softly and less compactly arranged than the other organs. The pancreas is divided into three parts, namely the head, body and tail; the head of pancreas is embedded in the curvature of the first loop of the duodenum while the tail region is tucked into the hilum of the spleen (Figure 1.1).

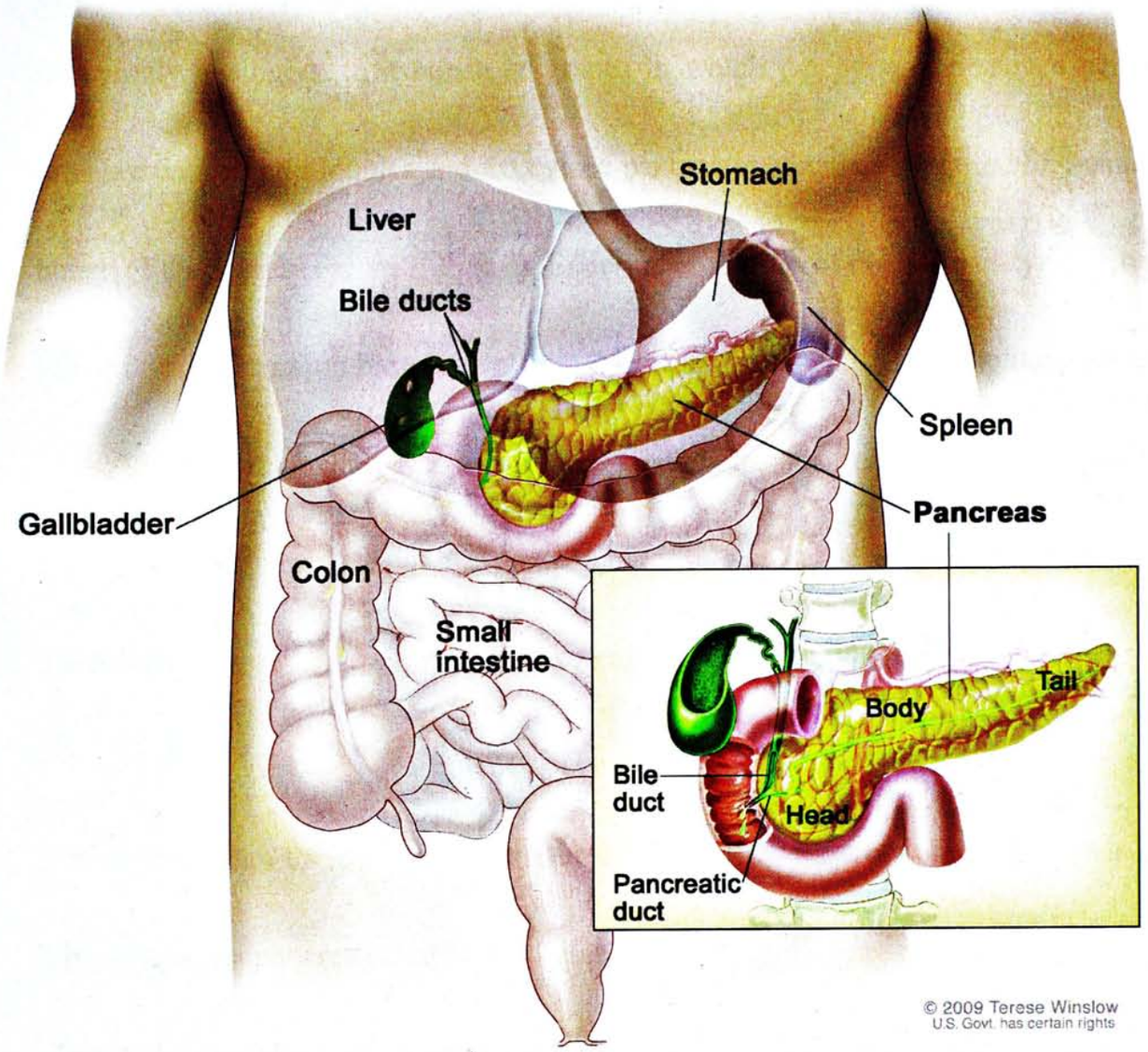

Figure 1.1 The macroscopic view of pancreas
[Extracted from: www.meb.uni-bonn.de/Cancernet/CDR0000062678.html]

The exocrine pancreas, consisting of pancreatic acinar cells and duct cells, is structurally analogous to a bunch of grapes in which the pyramidal-shaped acinar cells are packed into lobules and exocytose pancreatic enzymes into the blind-ended lumen of the acinus (Figure 1.2b & c). Exocrine secretions of digestive enzymes from acinar cells and sodium bicarbonate from ductal epithelial cells flow into intercalated ducts, larger intralobular ducts and major extrabobular duct, and finally drain to the main collecting duct that joins the common bile duct before entering into the duodenum (Figure 1.2a). (See review by Leung & Ip, 2006)

Embedded within these exocrine structures are small clusters of cells called Islets of Langerhans or pancreatic islets that make up the endocrine portion of pancreas (Figure 1.2d). Despite their small porportion in the pancreas (1-2% of total mass) compared with the endocrine pancreas (>80%), pancreatic islets are capable of producing a number of physiologically important peptide hormones including insulin, glucagon, somatostatin, pancreatic polypeptides, and ghrelin for the regulation of nutrient, notably glucose homeostasis. (See review by Kulkarni, 2004)

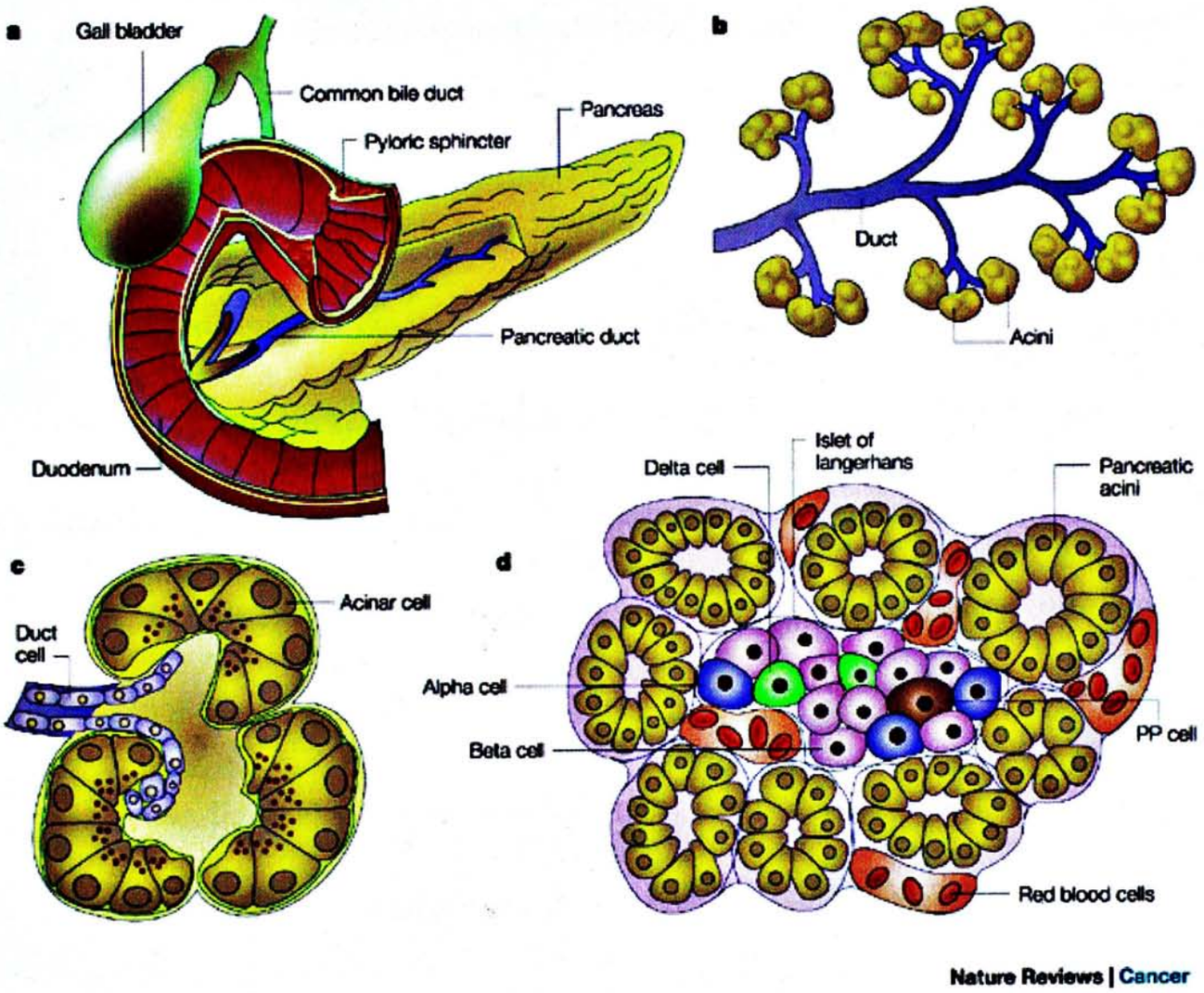

Figure 1.2 The microscopic view of pancreas, pancreatic duct, acinar and islet of Langerhan.

[Extracted from: Nabeel Bardeesy & Ronald A. DePinho. Pancreatic cancer biology and genetics. Nature Reviews Cancer 2, 897-909. 2002]

1.1.2 Structure and function of exocrine pancreas

As mentioned, the pancreatic exocrine gland, constituting over 80% of the total mass of the whole pancreas, is responsible for the production and secretion of digestive enzymes and an alkaline pancreatic juice. Such exocrine pancreatic secretions are finely regulated by two major hormones, secretin and cholecystokinin (CCK), which are released from the gut endocrine cells, i.e. D cells and K cells, respectively, in response to food stimuli. The pancreatic acinar cells are highly polarized cells, with a large basolateral membrane located at the acinar periphery and the apical membrane facing the acinar lumen. The acinar cells are capable of synthesizing and secreting over 10 different proteases or hydrolytic enzymes; they include to name, but a few, α-amylase, lipase, protease, ribonuclease, and deoxyribonuclease, all of which are the major digestive enzymes responsible for our daily food stuffs.

The secretion by the exocrine pancreas is modulated by neural and hormonal signaling pathways in the form of diverse neurocrines, paracrines and endocrines from the gastrointestinal system (Chey & Chang, 2001). For example, the neurocrine acetylcholine (ACh), the endocrine cholecystokinin (CCK) and secretin as well as the paracrine vasoactive intestinal polypeptide (VIP) and recently proposed angiotensin

II (Ang II) are physiologically important in the regulation of exocrine function (Leung & Ip, 2006). Among these regulators, CCK, one of these enteric endocrine hormones, plays an important role in governing the secretory function of exocrine pancreas. CCK is synthesized and released from the duodenal I cells under the stimulation of partially digested proteins and fats which are emptied from the stomach. Once released into the blood stream, CCK binds to the $G\alpha_q$ G-proteins-linked CCK_B receptor located on the cell membrane of pancreatic acinar cells, thus leading to the exocytosis of secretory granules via mediation of the phospholipase C (PLC)/Ca^{2+} signal transduction pathway (Wasle & Edwardson, 2002). On the other hand, the actions of VIP and secretin are mediated via their respective membrane-bound receptors and they activate $G\alpha_s$ G-proteins that finally lead to the polarized secretion of the digestive enzymes or exocytosis into the acinar lumen (Wasle & Edwardson, 2002). Interestingly, the vasoactive peptide, Ang II, has been recently proposed to play a role in regulating digestive enzyme secretions such as α-amylase and lipase from the pancreatic acinar cells (Tsang *et al.* 2004)

The ductal cells represent the minority of exocrine pancreas, comprsing only 10% of number of cells and 5% of total mass of the human pancreas (Githens, 1988);

however, are indispensable for the normal functioning of enzymes and the integrity of the duodenal mucosa. As such, they produce sodium bicarbonate (HCO_3^-)-rich alkaline fluid that provides optimal pH for pancreatic digestive enzymes as well as neutralizing gastric chyme which is emptied into the highly pH-sensitive duodenum. While secretin is the primary stimulus of HCO_3^- secretion by ductal epithelium, CCK is able to potentiate secretin-mediated HCO_3^- release. In addition, somatostain is released from the endocrine D-cells in response to gastric chyme (pH <3.5) entering the duodenum and thus inhibit various gastrointestinal secretions (Bayliss & Starling, 1902).

1.1.3 Structure and function of endocrine pancreas

1.1.3.1 Pancreatic islet and islet cells

As discussed, the endocrine pancreas is composed of islets of Langerhans or pancreatic islets, and their secretory products are physiologically critical for controlling glucose homeostasis. The islets are spherical in shape accounting only for 1-2% by volume of the total pancreatic mass. Each islet is richly supplied with blood vasculature, and the hormones that islet cells secrete enter these blood vessels directly. There are five major cell types which constitute the pancreatic islet, namely the alpha cells (α-cells), beta cells (β-cells), delta cells (δ-cells), PP cells (also known as F-cells) and, to some extent, the epsilon cells (ε-cells); these islet cells are responsible for the synthesis of glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively (Kulkarni, 2004). Among the five cell types, β-cells are predominant, consisting of about 80% of the total population of islet cells. Such cell type is so orderly arranged in the islets that β-cells are located in the centre closely surrounded by α- and δ–cells. Blood flows from the centre of the islet to the periphery in such a way that the α– and δ-cells are exposed to high concentrations of insulin, thus finely governing the release of glucagon and somatostatin in an autocrine manner. The distribution of these four

types of cells are uneven throughout the whole pancreas in which β- and δ–cells are distributed throughout the whole pancreas, while α-cells are located exclusively in the tail, body and superior part of the head of the pancreas; on the other hand, PP cells are present in the middle and inferior parts of head of pancreas (Wolfe-Coote & Du Toit, 1987).

Glucagon, a catabolic hormone, is exclusively produced by α-cells of which its major physiological action is to increase the plasma glucose levels by stimulating the synthesis of hepatic glucose via gluconeogenesis. The secretion of glucagon is activated by hypoglycaemic condition, high plasma levels of amino acids and epinephrine, as well as vagal activation. In addition, somatostatin inhibits glucagon release in a paracrine fashion. Generally speaking, glucagon is counteractive to insulin in terms of their physiological regulation on glucose homeostasis.

On the other hand, the function of β–cells is basically to synthesize and release insulin, an anabolic peptide hormone, in response to changes in plasma levels of major nutrients, particularly glucose, amino acids and fatty acids. The principal physiological action of insulin is to suppress levels of glucose via its uptake by

peripheral tissues such as adipose and skeletal as well as decreasing hepatic glucose production. Pre(pro)insulin, the precursor of insulin, is cleaved into (pro)insulin within the endoplasmic reticulum. The (pro)insulin consists of an amino-terminal β-chain, a carboxy-terminal chain, and a connecting peptide between these two chains called the C-peptide. The conversion of a mature insulin from (pro)insulin requires the removal of this C-peptide by the cleavage action of a specific endopeptidase known as prohormone convertase, which allows the exposure of the end of the insulin chain, thus rendering its interaction to insulin receptors (Steiner, 1997). The release of insulin is under regulation of numerous factors and they will be discussed in the following Sections.

1.1.3.2 Glucose-stimulated insulin secretion from islets

As mentioned in section 1.1.3.1, the release of insulin by β–cells is under the influence of changes in the plasma levels of the major nutrients. Apart from this, the insulin secretion can be up-regulated by several intestinal hormones such as insulin, glucagon-like-peptides (GLP-1), gastric inhibitory peptide (GIP), secretin and CCK, as well as by vagal and β-adrenergic stimulation. Insulin secretion is also under control of somatostatin and enhanced α-adrenergic activity, as well as physiological conditions like fasting and exercising (Widmaier et al., 2005).

β cells are capable of taking up metabolized glucose, galactose and mannose of each can provoke insulin secretion by islets. The mechanism that a glucose molecule stimulates the release of insulin can be explained by an established concept called glucose-stimulated insulin secretion (GSIS; see Figure 1.3). Glucose enters the β–cell via a membrane-bound glucose transporter, termed GULT2; once within the β-cell, glucose undergoes the chain reaction of glycolysis leading to the production of adenosine triphosphate (ATP). The resultant increase in the ratio of ATP to ADP inhibits the ATP-sensitive K^+ channel and thus the depolarization of K^+ channel activates the voltage-gated Ca^{2+} channel. Finally, a

surge of intracellular $Ca2^{+}$ results in exocytosis and release of insulin into the blood.

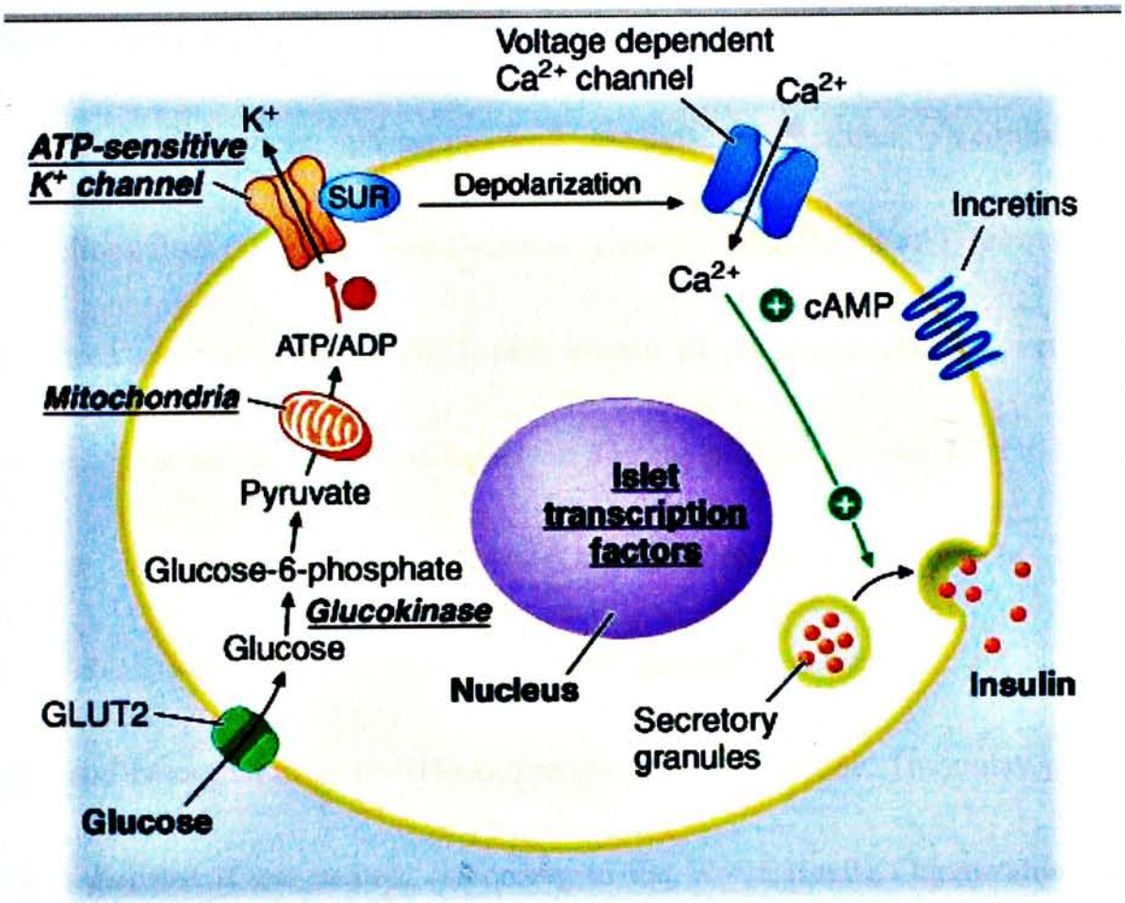

Figure 1.3 Schematic diagram of glucose-stimulated insulin secretion (GSIS) *[Extracted from Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J. Harrison's Principles of Internal Medicine, 17th edition. Http://www.accessmedicine.com]*

1.2Type 1 Diabetes

1.2.1 Pathophysiology of Diabetes Mellitus

Diabetes mellitus refers to a condition when the body fails to maintain normal blood glucose homeostasis. The prolonged high blood glucose level, which is also known as hyperglycemia would ultimately lead to diabetes mellitus. According to the guidelines from The American Diabetes Association (ADA, 2009), normoglycemia, impaired glucose tolerance (IGT, also known as pre-diabetes), and overt diabetes mellitus can be distinguished based on fasting plasma glucose (FPG) levels and 2-hour oral glucose tolerance test (OGTT). Details are shown in Table 1.1. Individuals with impaired FPG or IGT are generally euglycemic, but have normal glycated hemoglobin levels (HbA_{1c}) ranging from 3.5-5.5%. They may progress to overt diabetes if not treated. According to the World Health Organization (WHO), there are more than 220 million people worldwide having diabetes and this figure is likely to be more than double by the year of 2030 (WHO, 2009). In view of this, the epidemic of diabetes has a profound economic impact on our community. Patients with diabetes always experience polyuria, polydipsia and polyhagia, and these symptoms are concomitant with several chronic complications such as nephropathy, retinopathy and neuropathy, which eventually lead to renal failure, blindness and

diabetic foot, respectively.

Diabetes mellitus is generally classified as type 1 (T1DM), type 2 (T2DM) and gestational diabetes melitus (GDM; ADA, 2009). The two major types of diabetes, namely T1DM and T2DM account for approximately 10% and 90% of diabetes, respectively. T1DM, previously known as insulin-dependent or juvenile diabetes, is characterized by the immune-mediated destruction of insulin-producing islet β–cells in the pancreas, leading to insulin deficiency. Most of the T1DM are immune-mediated, with auto-antibodies against the islet cell antigens, islet cell surface antigens, GAD65 and GAD67. T1DM pathogenesis is thought to involve both genetic susceptibility and environmental factors. T2DM, previously known as non-insulin dependent or adult-onset diabetes, is characterized by the defective responsiveness of peripheral tissues to insulin or, more specifically, insulin resistance, along with reduced insulin secretion. T2DM represents the predominant form of diabetes among all cases. The etiology of T2DM is multifactorial and it is strongly associated with genetic and environmental disposition. The most frequent monogenic T2DM form is the autosomal dominant diabetes called maturity onset diabetes of the young (MODY). The majority of proteins associated with MODY are transcription

factors, such as hepatocyte nuclear factor 4α (HNF-4α), insulin promoter factor-1 (IPF-1) and NEUROD1 that directly or indirectly influence the expression of insulin genes (Malecki, 2005). There are so far six MODY identified, namely MODY 1,2,3,4,5 & 6, depending on the specific gene involved.

Condition	**2-hour Oral Glucose Tolerance Test (OGTT)** mmol/l(mg/dl)	**Fasting Plasma Glucose (FPG)** mmol/l(mg/dl)
Normal	<7.7 (<140)	<6.0 (<110)
Impaired Glucose Tolerance (IGT)	≥7.8 (≥140)& <11.1(<200)	≥ 6.0(≥110) & <7.0(<125)
Overt Diabetes Mellitus	≥11.1 (≥200)	≥7.0 (≥125)

Table 1.1 Diagnosis of pre-diabetes and diabetes by Fasting Plasma Glucose (FPG) and Oral Glucose Tolerance Test (OGTT). *[Information from: ADA, 2009]*

1.2.2 Autoimmunity in T1DM

T1DM is considered to be a chronic immune-mediated disease characterized by selective destruction of insulin-producing β-cells in the pancreatic islets. Studies have shown a subclinically prodromal period in these susceptible subjects. The cause of T1DM can be briefly classified into humoral and cellular autoimmunity of which the immune system is related. It has been widely accepted that cellular immune responses is the major mediator of β-cell destruction. Although the exact mechanisms involved in the initiation and progression of β-cell destruction are yet understood, reports revealed that β-cell autoantigens, macrophages, dendritic cells, B lymphocytes, and T lymphocytes are involved in the β-cell-specific autoimmune process (Rossini et al., 1993; Bach, 1995; Tisch & McDevitt, 1996; Delovitch & Singh, 1997; Yoon & Jun, 1999). Figure 1.4 outlines the possible etiology of T1DM. Autoreactive T cells, both $CD4^+$ and $CD8^+$ T cells, have been proved to be critically participated in β-cell destruction (Yoon & Jun, 2005). This is supported by the presence of autoreactive T cells in the circulation of patients with T1DM and the disease progression can be delayed by the use of T cell targeted immuo-suppressive drugs (Roep, 2003). There are two proposed mechanisms of cellular mediated β-cell death: the recognition-linked mechanism suggests that the direct T cell/β-cell contact

leads to the recognition of autoantigens by cytotoxic $CD8^+$ T cells and MHC molecules on the β cell surface, and alternatively, the activation-linked mechanism proposes that the recognition of antigens presented by MHC II molecules leads to the production of cytokines and death mediators by $CD4^+$ T cells.

On the other hand, the humoral immune system plays a role in T1DM. This is evidenced by the fact than more than 90% of T1DM patients have autoantibodies against one or several β-cell-specific autoantigens (Schranz & Lernmark, 1998) such as glutamic acid decarboxylase (GAD65), the protein tyrosine phosphatase-related islet antigen 2 (IA-2), insulin and, most recently, the zinc transporter Slc30A8 that is resided in the insulin secretory granule of the β cells (Todd *et al*, 2007). The most susceptible genes that contribute to the disease are located at the HLA class II locus on chromosome 6. However, additional factors are required to trigger and drive β–cell destruction in genetically predisposed subjects since only a small proportion of individual with HLA susceptibility develops into clinical diabetes (Knip & Siljander, 2008).

Despite the intensive investigations into the cause of autoreactive β-cell destruction,

the initial activation of autoreactive T-cells and/or the presence of the primary autoantigen remain largely controversial.

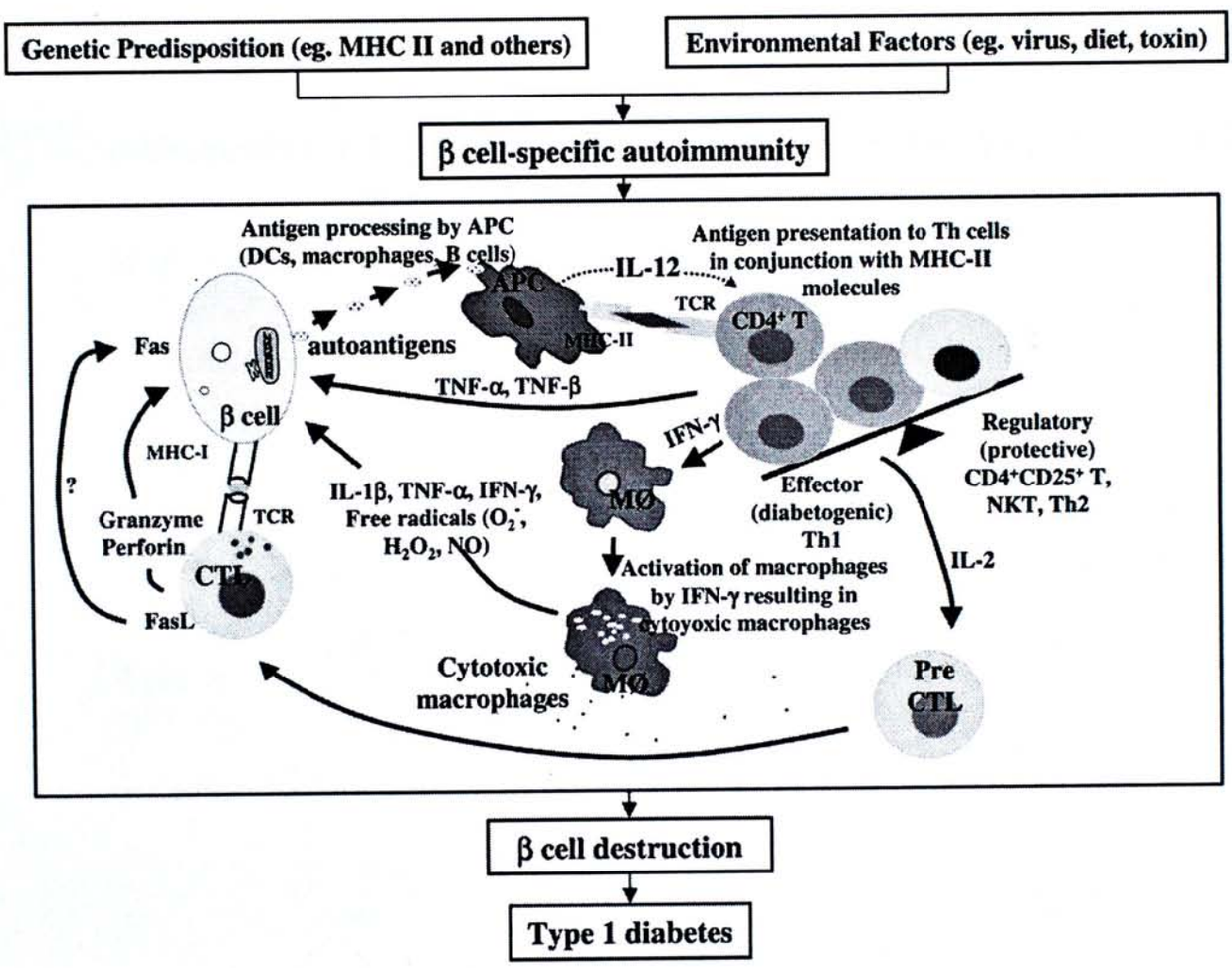

Figure 1.4 Etiology of T1DM

[Extracted from: Yoon JW, Jun HS. Autoimmune destruction of pancreatic beta cells. Am J Ther.;12(6):580-91. 2005]

1.2.3 Management of T1DM

1.2.3.1 Insulin replacement

Given the lack of endogenous insulin production in T1DM patients, the injection of exogenous insulin together with the strict monitoring of glycemia remains the current treatment for T1DM. While the intensive insulin regimen has been proved to be effective to delay and prevent disease progression to nephropathy, neuropathy or retinopathy (The Diabetes Control and Complications Trial, 1993), the intensification of insulin regimen actually leads to an increased risk of severe hypoglycemic episodes and low patient compliance with the intensive injection scheme. Therefore, the development of alternative therapies for T1DM is in desperate need to solve these problems as well as alleviating the long-term insulin dependence for patients with T1DM or severe form of T2DM.

1.2.3.2 Pancreas and islet transplantation

Given the challenges and limitations of exogenous insulin therapy, alternate solutions including whole pancreas and islet transplantation have been sought. Simultaneous pancreas and kidney transplantation is currently regarded as the standard therapy for T1DM patients with end-stage renal failure. Though pancreas transplantation achieves insulin independent in over 80% patients beyond a year (Sutherland et al., 2001), the risk associated with surgical procedures is always concerned. In contrast, islet transplantation is relatively safer than whole pancreas transplantation in terms of its non-invasive approach. A successful rate of 100% was noted in 7 long-term diabetic patients by the Edmonton Protocol in 2000, using a new immunosuppressive regimen and at least 10,000 islets obtained from a minimum of two donor pancreases (Shapiro et al., 2000). Patients having undergone islet transplantation have a remarkable improvement in life style which allows them to be independent from daily insulin injection and tight glycemic control. However, both pancreas and islet transplantation are hindered by life-long immunosuppression, organ scarcity and difficulties in islet isolation.

1.2.3.3 Stem-cell-based transplantation

While the shortcomings of pancreas and islets transplantation are yet to be overcome, the discovery of pluripotent stem cells as an inexhaustible source of precursor cells that can be differentiated into insulin producing cells provides the prospective for islet transplantation. Figure 1.5 highlights the generation of insulin expressing cells from different sources of pluripotent stem cells and organ specific progenitors. There are numerous potential sources of undifferentiated stem/progenitor cells that are capable of differentiating into insulin-secreting cells, including pancreas, liver, intestine, spleen, bone marrow and umbilical cord as well as embryonic stem cells (Lü et al., 2007).

The notion of the existence of pancreatic stem/progenitor cells within the pancreatic ductal cells, despite being contentious, is supported by substantial data. During embryogensis, stem cells within the pancreatic duct epithelium give rise to both endocrine and acinar cells of pancreas (Madsen et al., 1996) and the capacity to be expandable and differentiable still retain in adult pancreatic duct cells. Studies have demonstrated using pancreatic ductal epithelial cells extracted from non-obese diabetic (NOD) mice (Ramiya V.K., et al., 2000), neonatal rats (Ogata et

al., 2004) could produce insulin producing cells and reverse diabetes in different diabetic mouse models. Recently, the protocols for isolating, characterizing and culturing pancreatic progenitor cells (PPCs) from human fetus were reported (Hori *et al.*, 2008, Koblas *et al.*, 2008). A differentiation cocktail was avaliable to direct PSCs into hormone-producing ICCs (Suen *et al.*, 2008). With the propensity to differentiate and lower immunogenicity compared with adult tissues, PPCs become another promising source for stem cell transplantation.

Embryonic stem (ES) cells are also promising source for β cell. In this regard, some research groups have generated islet-like clusters from mouse ES cells through either a five-step (Lumelsky et al., 2001) or three-step (Schroeder et al., 2006) culture protocol. The clusters were glucose responsive and were able to exhibit vascularization upon transplantation. A normalization of glycemia was demonstrated in STZ-diabetic mice. However, achieving transplantable β–cells from ES cells is still in development and researchers are working to optimize the in *vitro* protocol. Prior to clinical application, it is also important to note the risks of teratoma formation from undifferentiated ES cells residues due to their oncogenic nature (Evans & Kaufman, 1981).

In addition, a subset of enteroendocrine cells called K cells was initially investigated for their ability to produce a glucose-dependent insulinotropic polypeptides that potentiate insulin release postprandially. In this context, it has been shown that using intestinal tumor cell line transfected to produce insulin can reverse the hyperglycemic condition of STZ-induced diabetic mice. (Han et al., 2007)

Stem-cell based therapy holds great promise in treating diabetes. However, there are controversies and challenges that have yet to be solved, especially the yet-to-be-modified protocol to generate fully mature and rich insulin-producing β-cells from different cell sources and the low efficacy of islet transplantation in terms of poor vascularization and immuno-rejection. Improvements on these aspects are likely to shape the future of cell-based diabetic therapy.

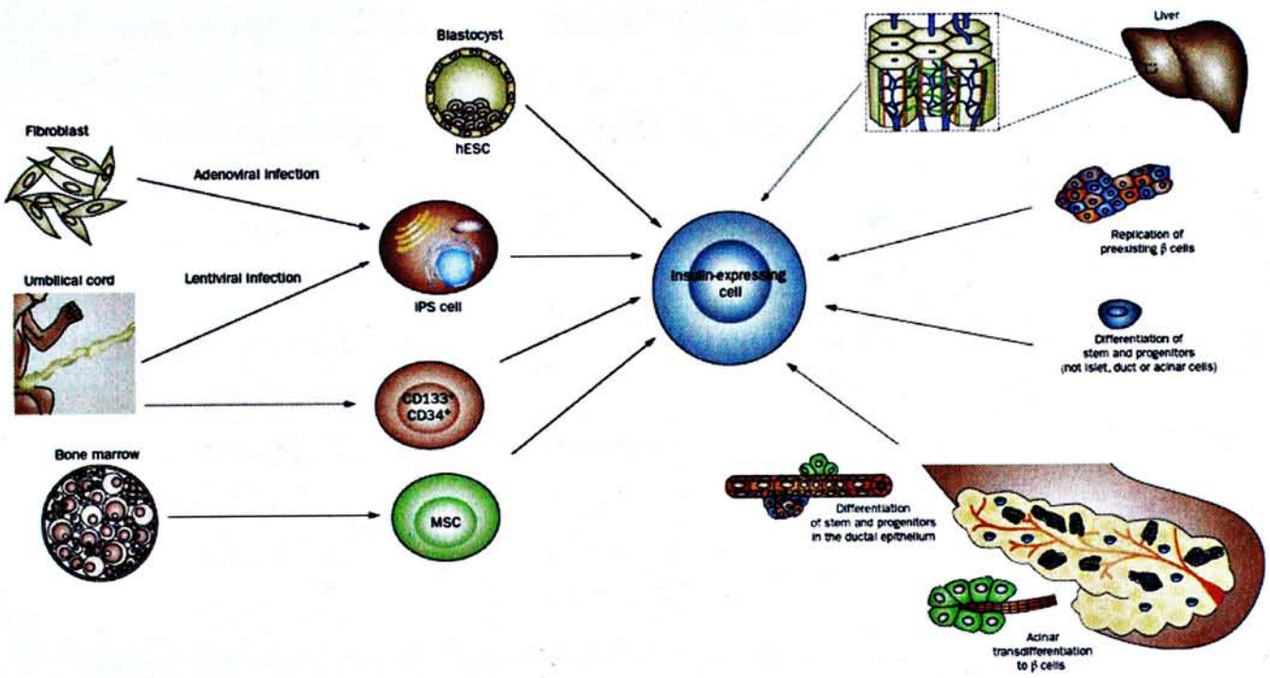

Figure 1.5 Generation of insulin-expressing cells from pluripotent cells and organ-specific progenitor cells.

[Extracted and modified from: Aguayo-Mazzucato C, Bonner-Weir S. Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol. 6(3):139-48. 2010]

1.3The adaptive immune system

1.3.1 T-lymphocytes

T lymphocytes, or T cells, is a type of white blood cells that has central role in cell-mediated immunity. The abbreviation "T" stands for the organ, thymus, in which T cells undergo maturation. T cells can be differentiated from other lymphocytes by its presence of unique T cell receptors (TCR) on the cell surface. Different subsets of T cells, including T helper cells, cytotoxic T cells, memory T cells, regulatory T cells, natural killer T cells as well as gamma delta T cells have been discovered which display respective processing distinct functions.

The major function of T helper cells, which is also known as $CD4^+$ T cells, is to assist other white blood cells in immunological processes including the maturation of B cells into plasma cells, as well as the activation of cytotoxic T cells and macrophages. T helper cells are activated upon the presentation of peptide antigens by the major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APCs). Once activated, T helper cells could differentiate into one of several subtypes, such as T_h1 and T_h2, that secrete different cytokines to facilitate different types of immune response.

On the other hand, cytotoxic T cells (also known as $CD8^+$ T cells) are rather destructive in nature that can destroy virally infected cells and tumor cells, and they are also responsible for transplant rejection. Naïve cytotoxic T cells are activated when their TCR recognized the antigens associated with MHC class I molecules. Upon activation, cytotoxic T cells undergo clonal expansion and travel throughout the body in search for cells bearing the target antigen. When exposed to the target cells, cytotoxic T cells release perforin and granulysin to lyse the cells. They also release granzyme that enters cells via pore to induce apoptosis. The activation of cytotoxic T cells is under a tight control and requires a strong activation signal from MHC molecules or helper T cells to prevent an extensive damage to cells and tissues.

The sole antigen-specific signal from the interaction between TCR and peptide-MHC molecules is actually inadequate to attain full activation of T cells. Indeed, a second signal, referred as the co-stimulatory signal, is provided by the interaction between co-stimulatory molecules on both APCs and T cells. CD28 is the co-stimulatory molecules expressed on T cells, which interacts with CD80 and CD86 on the membrane of APC for the full activation of T cell to proliferate and differentiate. (Figure 1.6)

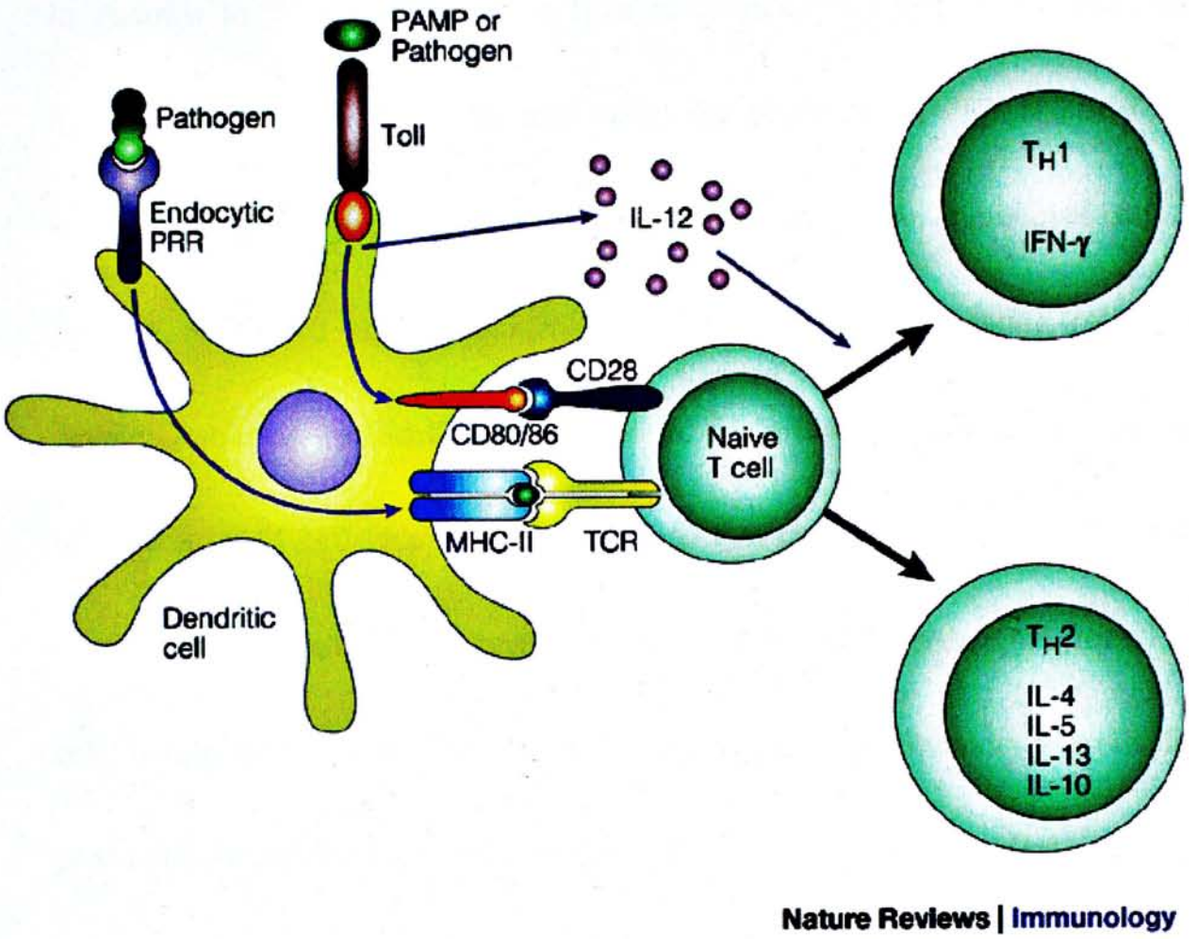

Figure 1.6 Costimulation in T-cells activation

[Extracted from: Medzhitov R. Toll-like receptors and innate immunity. Nat. Rev. Immunol. 1, 135–145. 2001.]

1.3.2 B-lymphocytes

In contrast to T cells, B cells are type of lymphocytes that play a main role in humoral immune response. In mammals, the abbreviation "B" stands for bone marrow where they are originally produced. The principal functions of B cells are to produce antibodies against antigens, perform as antigen-presenting cells, and to develop into memory B cells upon activation. Each B cells process a receptor protein, referred to as B cell receptor, on its surface that can bind to one specific antigen. Once B cells encounter its cognate antigen, with the additional signal from T helper cell, it can further differentiate into plasma B cells and memory B cells for the production of antibodies. Most activation of B cells is T-cell dependent, meaning that the activation requires the co-stimulation provided by T cells after B cells have ingested and processed an antigen and presented onto its own MHC molecules to the T cells. However, some antigens, such as the epitopes of bacteria, are T cell independent that they can deliver signals for B cell activation. The notion is demonstrated by the fact that athymic mice can respond to these antigens.

1.3.3 Major histocompatibility complex

1.3.3.1 Classification of MHC molecules

The immunological compatibility between donor and recipient is governed by the recognition of T cells to the molecules on the cell surface called major histocompatibility complex (MHC). The human leukocyte antigen (HLA) system is the name of the MHC in huma which is encoded on the short arm of chromosome 6 at 6p21.3 consisting of three major linked gene clusters, namely the class I, class II and class III regions. The class I region encodes three classical and most well known molecules HLA-A, B and C. Class I molecules are found to be expressed on most nucleated cells. In addition, three non-classical class I molecules HLA-E, F, and G are also encoded in the HLA class I region. In contrast to HLA-A, B and C, these non-classical class I molecules have distinct patterns of tissue expression. For instance, the expression of HLA-G is considerably high in trophoblast and is found to be important for protecting the fetus from the triggering of maternal immune response (Le Bouteiller, 2000). On the other hand, the class II region encodes the MHC class II molecules, including HLA-DR, DQ and DP. The expression of class II molecules is much more limited compared with class I, where expression is mainly distributed on professional APCs such as B cells,

macrophages and dendritic cells. The class III region comprises a diverse collection of genes such as complement components and tumour necrosis factors (TNF) α and β. Though numerous other genes are discovered in this region, their functions have yet to be confirmed.

1.3.3.2 Structure of MHC class I and II molecules

The structure of MHC class I and class II molecules are biochemically distinct on the basis of their subunits as shown in Figure 1.7. A MHC class I molecule consists of a large α-chain associated non-covalently with the smaller β_2-microglobulin, with which only the α chain spans the membrane. Whereas a MHC class II molecule consists of two transmembrane glycoprotein chains, α and β, both of which are non-covalently associated with the cell surface. The structures of MHC class I and class II molecules are similar in such a way that each molecule contains an extracellular portion composed of four domains. The membrane distal domains fold together to form a long groove while the rest of the extracellular portion adopts an immunoglobulin-domain-like structure. Despite their similarity, there are many minor structural differences that render the difference in the way they interact with peptide antigens.

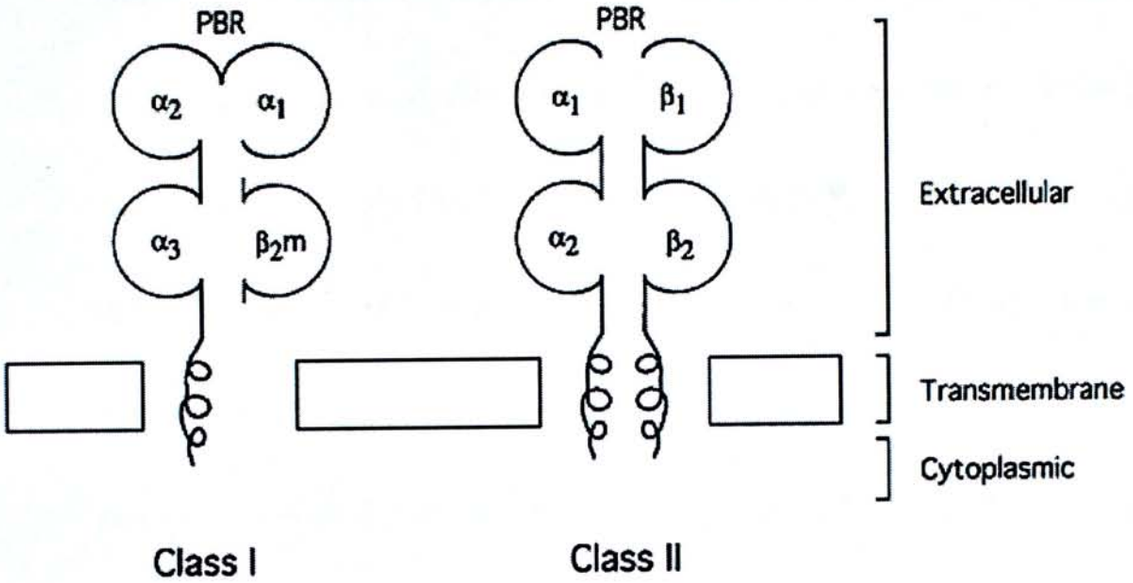

Figure 1.7 Structure of MHC class I and II molecules. PBR=protein binding region. *[Extracted from: the Annual Review of Genetics, Vol. 32 ©1998 by Annual Reviews, www.annualreviews.org]*

1.3.3.3 Function and regulation of MHC molecules

The central role of MHC molecules is the presentation of antigens to T lymphocytes. Unlike B cells which recognize the native form of antigen, T cells are specific for the recognition of short peptide fragments that are derived by the proteolytic cleavage of intact antigens, and which are then presented by MHC molecules to T cell receptor (TCR) (Bevan, 1975). Hence the antigen recognition of T cells is restricted to MHC and the antigen peptide that it presents. Two forms of mature T cells, namely $CD4^+$ and $CD8^+$ T cells are restricted to MHC class II and class I molecules, respectively.

The two major classes of MHC molecules possess very different patterns of tissue distribution. MHC I molecules are widely expressed on different tissues. As mentioned above, the expression of MHC II molecules are much stricter than that of class I. MHC class II are expressed exclusively on professional APC such as B lymphocytes, macrophrages and dendritic cells. Nevertheless, MHC Class II expression can somehow be induced in tissues which are otherwise negative for class II under pathological conditions. For instance, in patient with T1DM, class II expression was observed in β-cells of the pancreatic islet tissue

(Bottazzo et al 1985). Meanwhile, the expression of class I and II genes can be induced or up-regulated by certain immune mediators such as interferons (Collins et al. 1984; Pujol-Borrell et al. 1987) and TNF-α (Collin et al. 1986; Arenzana-Seisdedos et al. 1988).

1.3.4 HLA-G and its immuno-modulatory properties

HLA-G is the best studied of the three non-classical MHC molecules that play a key role in the induction of immune tolerance. It is categorized as non-classical because of their difference with classic MHC I molecules by its genetic diversity, expression, structure and functions (Carosella et al., 2008). HLA-G has a very low polymorphism with only 8 protein variants compared with 462 and 789 for HLA-A and -B, respectively (Carosella et al., 2008). On the other hand, the expression of HLA-G is highly restricted to fetal trophoblast cells (Kovats et al., 1990), and is constitutively expressed in adult thymic medulla (Mallet et al., 1999), cornea (Le Discorde et al., 2003), pancreatic islet (Cirulli et al., 2006) and erythroid and endothelial-cell precursors (Menier et al., 2004). Expression of HLA-G is induced in several pathological conditions such as cancers, transplantation, multiple sclerosis, inflammatory disease and viral infections (Carosella et al., 2008). The HLA-G primary transcript is spliced in seven messenger RNAs (mRNAs) that encode four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three soluble (HLA-G5, -G6 and –G7) protein isoforms (Figure 1.8).

Given the nature of trophoblast that makes up the physical maternal-fetal interface, the localized HLA-G expression on placental trophoblast cells implies a critical role in the

maternal immune accommodation of the semi-allogeneic fetus. Table 1.2 depicts the inhibitory effect of HLA-G on different types of cells. The underlying mechanism lies in the capability of HLA-G to bind inhibitory receptors, called the leukocyte immunoglobulin-like receptors (ILTs), that interferes with leukocyte activation. HLA-G molecules are capable of inhibiting the cytolytic function and proliferation of NK cells and cytotoxic T lymphocytes as well as the maturation and function of dendritic cells (Carosella et al., 2008). The expression of HLA-G protein was previously demonstrated in some human melanoma cell lines. Several reports have demonstrated the capacity of interferons, GM-CSF, IL-2 and IL-10 in the enhancement of HLA-G protein expression (Carosella et al., 1999).

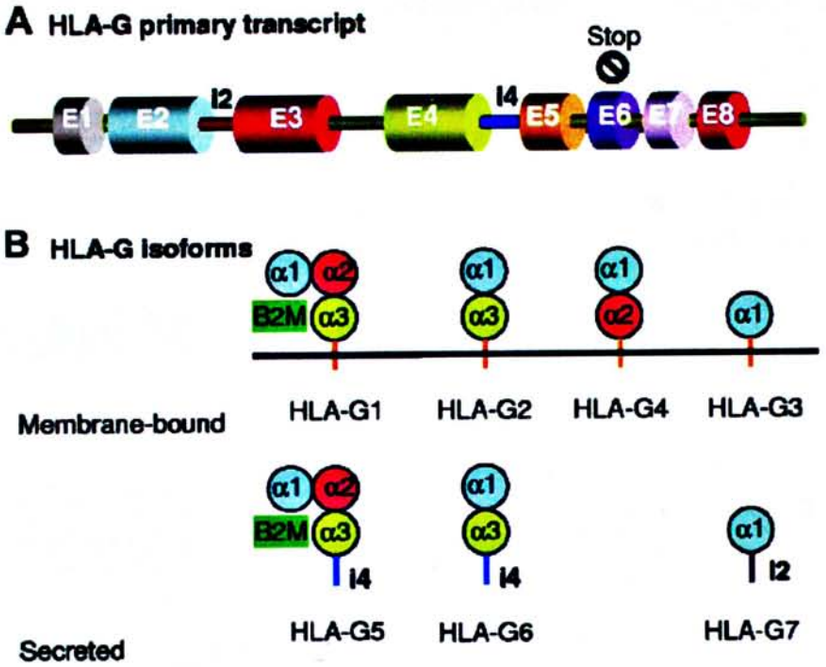

Figure 1.8 Primary transcript and isoforms of HLA-G

[Extracted from: Carosella ED, Favier B, Rouas-Freiss N, Moreau P, Lemaoult J. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. Blood. 111(10):4862-70. 2008]

Effector cell	HLA-G function
NK cells	Inhibition of cytotoxic function
	Indirect inhibition of cytotoxic function through stabilization of cell-surface HLA-E
	Inhibition of proliferation
	Up-regulation of inhibitory receptors
	Apoptosis
	Increased proliferation and IFN-γ production
	Increased secretion of pro-angiogenic factors
	Inhibition of transendothelial migration
$CD8^+$ T cells	Inhibition of cytotoxic function
	Inhibition of proliferation
	Generation of CD8-low regulatory T cells
	Apoptosis
$CD4^+$ T cells	Inhibition of alloreactivity
	Inhibition of proliferation
	Up-regulation of inhibitory receptors
	Generation of regulatory T cells, including CD4-low T cells
APC	Inhibition of dendritic cells maturation, antigen presentation, trafficking, and induction of regulatory T cells
	Up-regulation of inhibitory receptors
PBMC	Secretion of Th2 cytokines
Endothelial cells	Apoptosis

Table 1.2 The inhibitory effect of HLA-G on various kinds of cells. *[Extract and modified from Carosella ED, Favier B, Rouas-Freiss N, Moreau P, Lemaoult J. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. Blood. 111(10):4862-70. 2008]*

1.4Transplantation rejection

1.4.1 Mechanisms involved in transplantation rejection

Allogeneic grafts are inevitably subjected to immune rejection triggered by both the innate and adaptive immune system. The immune responses is mediated by the disparate antigens present on the donor tissues or cells, which can be categorized into three major groups including human ABO blood group antigens, HLA molecules and the minor histocompatibility complex (mHC; Bradley et al., 2002). The ABO antigens are expressed on almost every cell in the body. The transplantation of vascularized organs between incompatible individual can lead to hyperacute rejection, which is mediated by antibodies directed toward the ABO-incompatible antigens. Although the expression of ABO antigens on stem cells are not known, it is advised to avoid ABO imcompatibility when transplanting stem-cell derived products, especially for non-vascularized tissue allografts such as pancreatic islets (Bradley et al., 2002).

Apart from ABO antigens, HLA-disparities between donor-recipient pair should be the most formidable immunological barrier to transplantation. The central role of MHC molecules is the presentation of antigens to T lymphocytes for triggering

immune responses. Minor histocompatibility complex (mHC) antigens are comparatively less remarkable but their effects cannot be completely discounted as they may initiate immune responses when exist in large number (Simpson et al., 2001).

1.4.2 Immunobiology of rejection

Allorecognition refers to the ability of recipient T cells to recognize donor-derived antigens that initiates immune responses. This can be divided into two distinct pathways, namely the direct and indirect pathway (as shown in Figure 1.9). Both depend on the presentation of donor cell antigens. In the direct pathway of allorecognition, T cells recognize intact non-self MHC molecules present on the surface of donor cells; whereas indirect pathway describes the ability of T cells to recognize donor MHC molecules that are processed and presented as peptides by self-MHC molecules.

1.4.2.1 Direct allorecognition pathway

In the direct recognition (Figure 1.9 left), the APCs that exist in the donor tissue, upon transplantation, migrate to the nearest lymphoid system to trigger host immune response. Evidences have shown that dendritic cells (DCs) are the passenger leukocyte responsible for the induction of an acute anti-allograft response (Fung et al., 1985). DCs are abundant within peripheral tissues and organs in their mature state positioned for antigen capturing. These cells would undergo a mature process upon the stimulation of inflammatory signals such as interleukin (IL-1β) and tumor necrosis factor (TNF-α), and migrate to the lymph nodes for the activation of naive memory T cells (Archbold et al., 2008). T cells mistakenly interpret the donor MHC/peptide complex on DCs as self-MHC with a foreign peptide, and together with co-stimulation, resulting in T cell activation (Grinnemo et al., 2008). The graft-specific T cells then infiltrate to the graft by recognizing directly to the alloantigens on the graft parenchyma. Direct antigen presentation is the pathway that induces the strongest immune responses. Nevertheless, cellular transplant usually do not contain donor APCs and the rejection of cellular grafts are less likely to be induced by direct recognition pathway (Grinnemo et al., 2008).

1.4.2.2 Indirect allorecognition pathway

The concept of indirect allorecognition pathway (Figure 1.9 right) were proposed upon the rationale that the DC population in the transplanted graft would decline with time and that chronic rejection can only be explained by an indirectly pathway in which recipient DC take up and process alloantigens and then present it via self-MHC class II molecules to $CD4^+$ cells in local lymphoid tissues (Caballero et al., 2006). Some antigens of the grafts may also be presented to $CD8^+$ T cells via class I MHC pathway. (Grinnemo et al., 2008).

1.4.2.3 Semi-direct allorecognition pathway

Apart from that generation of antigen-specific $CD8^+$ cells requires the activated $CD4^+$ T cells, climbing researches have demonstrated the participation of DCs in the allorecognition responses. Evidences have shown that MHC molecules can be transferred between cells, the recipient DCs are capable of taking up and presenting intact donor MHC class-I molecules to directly stimulate $CD8^+$ T cells, and at the same time internalized and process donor MHC molecules as peptides to $CD4^+$ T cells with indirect allospecificity. The exploration of this pathway has provided an alternative explanation on the mechanism of rejection

of grafts derived from stem cells that are usually devoid of DCs but not necessarily show reduced rejection response.

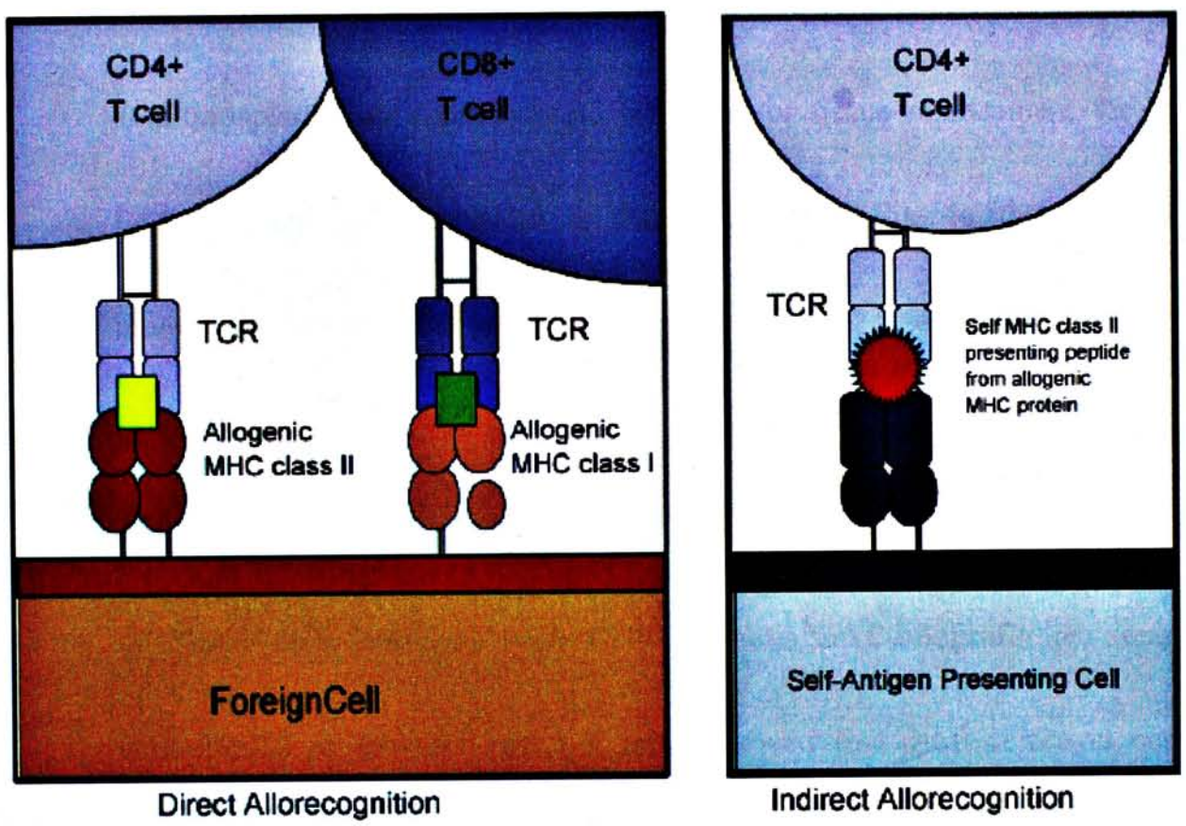

Figure 1.9 Direct and indirect T-cell recognition pathways.
[Extracted from: Archbold JK, Ely LK, Kjer-Nielsen L, Burrows SR, Rossjohn J, McCluskey J, Macdonald WA. T cell allorecognition and MHC restriction--A case of Jekyll and Hyde? Mol Immunol. 45(3):583-98. 2008]

1.4.3 Xenotransplantation

Xenotransplantation is the transplant of organ or tissue from one species to another. Though it might seem impossible in first sense, the highly successful incidence in transplantation of porcine heart valves has proved xenotransplantation a new ground for organ or tissue replacement for human. Unfortunately, xenotransplant is often dangerous due to the high risk of incompatibility of tissue antigens, rejection and viral infection from one species to another. Although the attributes to xenorejection are numerous, the cellular response is primarily T cell mediated (Sachs et al., 1990). In a study, the transfer of blood cells enriched with $CD4^+$ T cells into allografts or xenografts transplanted to athymic BALB/c mice showed that graft rejection occurred sooner in xenotransplant than in allotransplant, whereas the transfer of $CD8^+$ T cells were ineffective in rejecting either types of grafts. Data suggest that xenorejection is $CD4^+$ T cell dependent, and its mechanism is distinctive from that of allograft rejection (Mandel, 1999). The selective dependence of xenorejection on CD4+ T cells were also demonstrated in other models using skin (Pierson et al., 1989) and pig islet tissues (Mandel et al., 1990; Mandel et al., 1990; Simeonovic et al., 1990). However, the mechanisms by which $CD4^+$ T

cells destroy xenografts are yet understood (Morris et al., 1995; Yi et al., 2002).

1.5 Cytokines and immunity

1.5.1 Interferons

Interferons (IFNs) is a kind of glycoproteins originally discovered as an agent that interferes with viral replication (Isaacs & Lindenmann, 1957). They are produced by cells of the immune system in response to challenges by foreign agents such as viruses, parasites and tumor cells. There are three main types of IFNs, including IFN type I, II and III, by which the classification is accorded with their receptor specificity and sequence homology. It is surprising to observe that IFNs, instead of being an antiviral molecule alone, are actually capable of inducing massive de-repression of the cellular genome and stimulating expression of a large number of cellular genes of many different functional categories. This indicates that IFNs participate in a diversity of cellular functions and many of the mechanisms have yet to be elucidated.

1.5.1.1 Interferon-γ and its immune regulation

IFN-γ is designated as "immune" or type II IFN and is produced by leukocytes.. Though both type I and II of IFN are crucial in the immediate cellular response to viral infection, IFN-γ is more important in coordinating the later immune response and establishing an antiviral state for longer term control.

IFN-γ is able to orchestrate the trafficking of specific immune cells to the site of inflammation through up-regulating the expression of adhesion molecules and chemokines. At the site of inflammation, IFN-γ and NO are produced, causing local dilation of blood vessels, thus decreasing the local blood flow. Hence, the unstimulated leukocytes that are originally cycling between blood and lymph would gather at the leaky vessels. Many reports has suggested that IFN-γ arrests the macrophage cell cycle and provides a survival signal, while other suggested that it serves as a pro-apoptotic signal (Schroder et al., 2004). The antiviral, antiproliferative and immunoregulatory effects the IFN-γ exerts on cells were believed to be mediated by a set of genes induced by the cytokines. Table 1.3 showed different categories of genes or proteins regulated by IFN-γ. The primary signaling pathway activated by IFN-γ has been known as Janus Kinase

(JAK)-STAT pathway. Given the complexity and diversity of IFN-γ as an immune modulator, we would therefore focus on its effects on the expression of MHC molecules as well as the regulation of IFN-γ production upon stimulation.

Gene/protein up-regulated by IFN-γ	Function
MHC antigen Processing and Presentation	
Class I MHC heavy chain	The heavy chain associates with β2-microglobulin to form the MHC class I complex (MHC I). MHC I displays foreign and self-peptides on the cell surface for immune surveillance by cytotoxic T cells. The class I heavy chain is encoded by the class I MHC locus.
PA28 α, PA28 β	Proteasome activator (PA)28α:PA28 β dimer is a nonenzymatic proteasome subunit, which alters the specificity of peptides generated to increase efficiency of class I MHC peptide delivery.
α1, α2, β1, β2 MHC II chains	Constituents of the heterodimeric MHC II. MHC II displays foreign and selfpeptides on the cell surface for immune surveillance by $CD4^+$ T cells. The MHC II α and β chains are encoded by the class II MHC locus.
Cathepsins B, H, L	Lysosomal proteases implicated in peptide production for class II MHC loading.
Activation of Microbial Effector Functions	
iNOS/NOS2	The NOS enzymes (NOS1, iNOS, NOS3) catalyze the reduced nictonimide adenine dinucleotide phosphate NADPH)-dependent conversion of L-arginine to L-citrulline, forming NO as a by-product. Of these, iNOS is the only isoform inducible by cytokine and/or microbial stimulus.
C2, C4, Factor B	Complement proteins are secreted by macrophages and fibroblasts in response to IFN-γ. Complement functions to opsonize extracellular pathogen for receptor-mediated phagocytosis by mononuclear phagocytes.

Immunomodulation and Leukocyte Trafficking	
ICAM-1	Adhesion molecule-binding to lymphocyte function-associated antigen-1 and Mac1.
Vascular cell adhesion molecule-1 (VCAM-1)	Adhesion molecule-binding to very late antigen-4.
B7.2	Surface molecule on APCs that provide a costimulus for antigen-specific T cell activation.
MIP-1α, MIP-1β (CCL3, CCL4)	Chemoattractant for $CD4^+$, $CD8^+$, and memory T cells.

Table 1.3 Some highlighted genes or proteins regulated by IFN-γ. *[Extract and modified from Schroder K, Hertzog PJ, Ravasi T, Hume DA.Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 75(2):163-89. 2004].*

1.5.1.2 Effect and kinetics of interferon-γ on MHC molecules expression

IFN-γ has been discovered to be involved in the up-regulation of both MHC class I and II molecules. This is of crucial significance to increase the quantity and diversity of peptides presenting on cell surfaces and thus potentiating the antigen recognition of T cells in order to induce cell-mediated immune response upon pathogen invasion. Regardless the cognate of MHC I and II molecules, the kinetics of IFN-γ induced expression of the two classes are different in terms of the signaling molecules involved.

The known IFN-γ signal transduction pathway inducing the expression of MHC I and II molecules are demonstrated in figure 1.10. Upon the binding of IFN-γ to its receptors, which is comprised of IFN-γR1 and IFN-γR2, the signal transducers and activators of transcription-1 (STAT-1) is phosphorylated by the activated JAK1/2, followed by STAT-1 homodimerization. The dimerized STAT-1 then translocates to the nucleus to bind to the IFN-γ activation site (GAS) to initiate the transcription of interferon regulatory factory-1 (IRF-1). For the induction of MHC class I molecules, IRF-1 regulates the transcription of interferon-stimulated genes (ISGs) on IFN-stimulated response elements (ISRE),

leading to the transcription of several MHC class I related genes. On the other hand, apart from inducing IRF-1, the dimerized STAT-1 itself also binds onto the GAS promoter regions to induce the transcription of CIITA. Its protein would induce the MHC-II expression on cell surface for the activation of T cells.

The IFN-γ inducible components are not restricted only to the MHC molecules itself but also genes that are related to the processing and presentation of peptides. For instance, apart from the induction of the expression of heavy chain and the light chain of MHC-I (Decker et al., 2002), IFN-γ induces the new subunits, LMP2, MCL-1 and LMP7, which can competitively replace the original subunits of the proteasome (Belich et al., 1994; Hisamatsu et al., 1996; Nandi et al., 1996). This proteasome replacement is one of the suggested mechanisms to increase the quantity, quality and repertoire of peptides for class I MHC loading. Likewise, some accessorizing molecules, such as the lysosomal proteases cathepsins which functions to produce antigenic peptides for class II MHC loading, are also induced by IFN-γ.

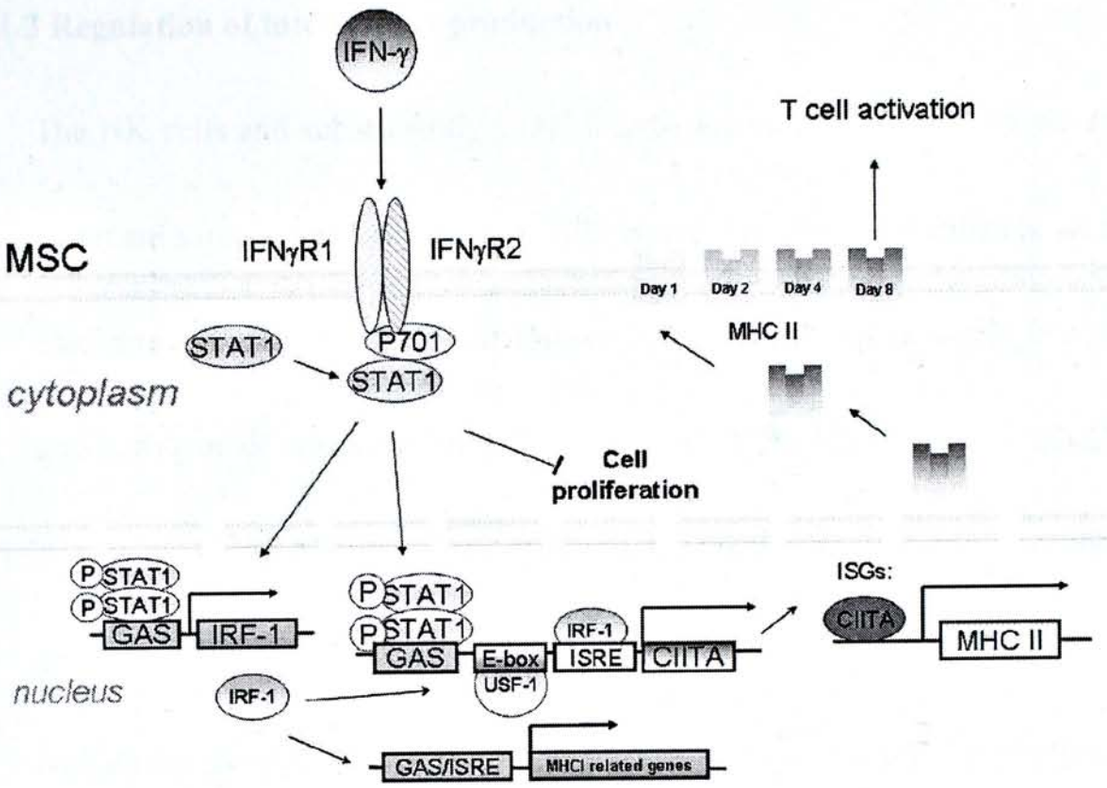

Figure 1.10 MHC I and II induction through IFN-γ signal transduction pathway
[Extracted from: Chan WK, Lau AS, Li JC, Law HK, Lau YL, Chan GC. MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. Exp Hematol. 36(11):1545-55. 2008]

1.5.1.3 Regulation of interferon-γ production

The NK cells and subsequently $CD4^+$ T cells are classically well known as the two main sources to produce IFN-γ. Although the precise mechanism is yet to be elucidated, it was proven that the transcription factors, such as signal transducer and activator or transcription 4 (STAT-4) and T-bet (Frucht et al., 2001) are involved. Briefly (Figure 1.11a), APCs such as dendritic cells and macrophages produce interleukin-12 (IL-12) to induce the production of IFN-γ by NK cells and direct the differentiation of naïve $CD4^+$ T cells into IFN-γ producing T helper 1 (Th1) cells (Trinchieri et al., 1998). IFN-γ, in turn, enhances the antigen-presentation of DCs and macrophages to promote the killing of intracellular pathogens in the macrophages. This positive feedback crosstalk between CD4+ T cells and APCs allows the potent response to infection.

Recent studies have also suggested the production of IFN-γ by APCs themselves (Frucht et al., 2001; figure 1.11b), in which the production of IL-12 and IL-18 synergistically induce the production of IFN-γ in an autocrine manner in these APCs, thus contributing to the enhancement of immunity against pathogens before the specific recognition by Th1 cells. Meanwhile, several cytokines have

been found to down-regulate the production of IFN-γ, these include IFN-α, IL-4 and IL-10.

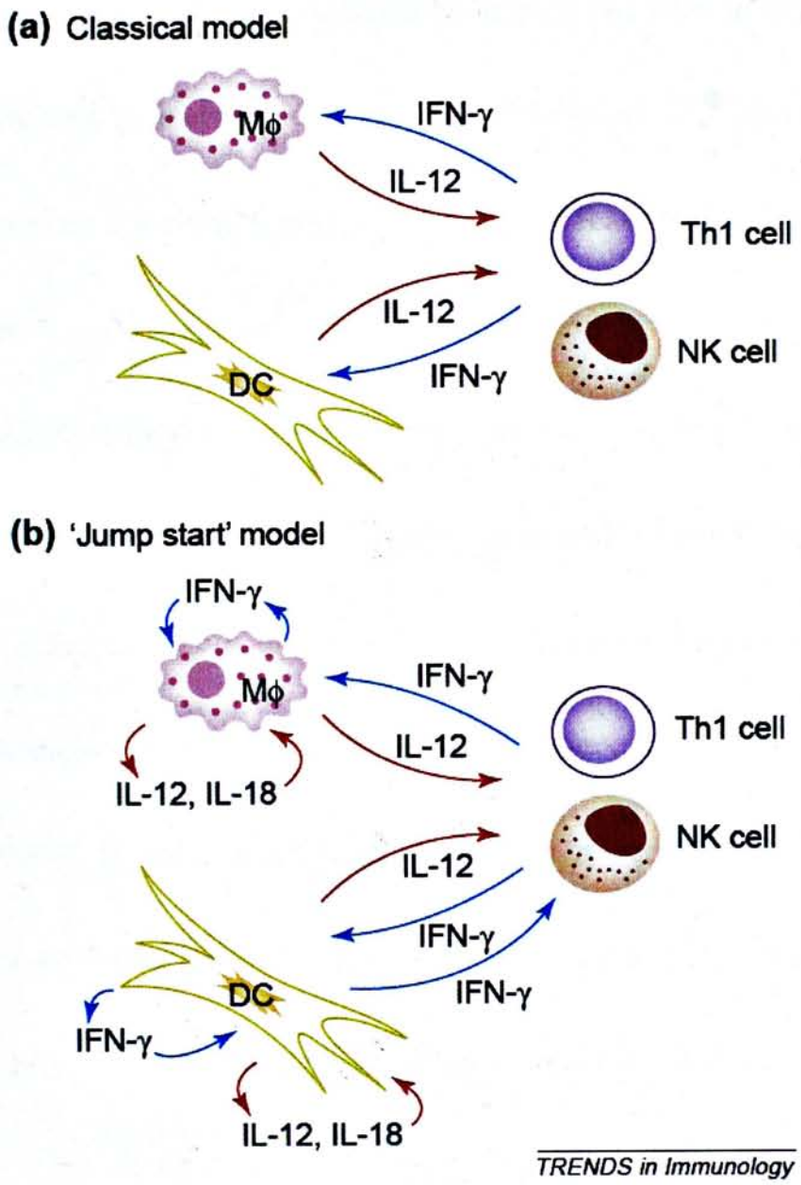

Figure 1.11 Classical and "jump start" model of production of IFN-γ by immune cells.*[Extracted from: Frucht DM, Fukao T, Bogdan C, Schindler H, O'Shea JJ, Koyasu S. IFN-gamma production by antigen-presenting cells: mechanisms emerge. Trends Immunol. 22(10):556-60. 2001]*

1.5.2 Interlukins

1.5.2.1 IL-10 and its immune regulation

IL-10 is first described as cytokine synthesis inhibitory factor (CSIF; Fiorentino et al., 1989), which is produced by murine Th2 cells to inhibit the activation and cytokine production of Th1 cells (Moore et al., 2001). Later studies have revealed the broad inhibitory spectrum of IL-10. Whilst the ultimate function of IL-10 is to suppress inflammatory responses to prevent damage to host, this multifunctional cytokine actually possesses diverse effects on most hemopoietic cell types, including macrophages, monocytes, dendritic cells, NK cells, B cells, T cells, mast cells and granulocytes. IL-10 potently inhibits the cytokines and chemokines production in activated monocytes/macrophages (Moore, et al., 2001). It was noted that IL-10 suppresses the production of IFN-γ in macrophages (Flesch, et al., 1994). IL-10 also acts on macrophages and DCs to inhibit the activation of Th1 cells (Moore, et al., 2001).

In addition, it was revealed that IL-10 plays a key role in the differentiation and function of a newly appreciated type of T cells, termed as the T regulatory cells (Treg), which itself produce IL-10 and function to inhibit Th1 and Th2-mediated

immune responses. The relationship adds on the complexity of the immuno-inhibitory profile of IL-10 (Roncarolo et al., 2006).

1.5.2.2 IL-10 and HLA-G

Evidences have shown that there is an interactive relationship between IL-10 and HLA-G, yet the whole picture has not been clearly elucidated. Hypothesis lies in the fact that these two molecules are working closely together to perform certain immuno-suppressive effects. The capability of HLA-G to modulate cytokine production is widely investigated. Recently, HLA-G^+ Treg cells were identified in the peripheral blood of healthy individuals that is enriched at the site of inflammation (Feger et al., 2007; Huang et al., 2009). It has been demonstrated that the secretion of IL-10 is elevated in HLA-G^+ Treg cells when compared with HLA-G^- cells. The suppressive effect mediated by HLA-G^+ Treg cells is IL-10 dependent (Huang et al., 2009). Some other studies also showed that the incubation of peripheral blood mononuclear cells (PBMCs) with either purified HLA-G (Kapasi et al. 2000) or soluble HLA-G (Kanai et al., 2001) results in the increased production of IL-10 by PBMCs. Nückel H. et al. showed that the plasma levels of IL-10 tended to be higher in patients with HLA-G^+

leukemias than in patients with HLA-G$^-$ leukemias (Nückel H. et al., 2005). The observations lend support to the potential regulatory effect of IL-10 production by HLA-G.

Likewise, emerging data have suggested that IL-10 plays a role in regulating the expression of HLA-G on several cell types. It was observed in trophoblasts and monocytes that the expression of HLA-G can be induced by IL-10 in a regulatory pathway separated from other MHC class I genes (Moreau et al., 1999). An upregulation of HLA-G by IL-10 is also observed in decidual stroma cells (Blanco et al., 2008) and cutaneous lymphomas (Urosevic & Dummer, 2003). Hence IL-10 can regulate the HLA-G expression, and the regulation runs in an autocrine as well as a positive feedback manner. The correlation between HLA-G and IL-10 was also observed in stem cells, as evidenced by the inhibitory effect towards T cells and NK cells, and an induction of Tregs in bone marrow-derived MSCs was mediated through the production of soluble HLA-G5 in an IL-10-dependent manner (Selmani, et al., 2008). The readouts leave room for the investigation on the existence and function of this HLA-G/IL-10 axis in other type of stem cells.

1.6 Stem cells and their immunogenicity

Despite being immuno-privileged compared with whole tissue transplant, the major obstacle of stem cells and their derivatives in the clinical use still rests on the immune response towards the transplanted cells. The immunological properties of different stem cells and the strategic manipulations to circumvent immune rejection attract much attentions.

1.6.1 Embroynic stem cells

Human embryonic stem cells (hESC) are pluripotent stem cells isolated from the inner cell mass of blastocysts. Differentiation protocols for many cell types, such as insulin producing islet cells (Kroon et al., 2008), neurons (Johnson et al., 2007; Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001; Schulz et al., 2003) and endothelial cells (Levenberg et al., 2002) are currently available. This implies the potential of hESC to treat diseases such as diabetes and Parkinson's disease.

Undifferentiated hESC express low levels of MHC class I and no MHC class II molecules, whereas the spontaneous differentiation of hESC *in vitro* into embryoid bodies (EBs) achieved an increase in expression of 2-4 folds (Drukker et al., 2002).

Nonetheless, the expression was still below those for other somatic cells. The addition of cytokine IFN-γ could considerably increase MHC-I expression (Drukker et al., 2002; Li et al., 2004; Grinnemo et al., 2006), while neither IFN-α nor IFN-β had any effect. Similar stimulatory effects on MHC-I molecules were also observed in differentiated hESC (Andrews et al., 1987). In contrast to MHC-I, MHC-II molecules were absent in both undifferentiated and differentiated hESCs even in the present of IFN-γ (Drukker et al., 2002). It was noted that the expression of co-stimulatory molecules, namely B7.1, B7.2 and CD40, are also low or absent on undifferentiated hESC (Li et al., 2004; Grinnemo et al., 2006). In a mixed lymphocyte reaction (MLR), hESC failed to induce the proliferation of human PBMCs (Li et al., 2004). When hESC were injected into the myocardium of immunocompetent C57BL/6 mice, signs of rejection were observed within 3 days, which peaked at day 5 to 7 (Grinnemo et al., 2006).

1.6.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are pluripotent stromal cells that can be found in virtually all postnatal tissue. Since MSCs can give rise to diverse lineages of cell types, the notion of MSC-based cell therapy has become an emerging strategy for the replacement of injured tissues. A more interesting aspect about MSCs is their ability to induce profound immunomodulatory effects in vivo, and therefore these cells have been extensively exploited in clinical trials to reduce the burden of immune-mediated disease such as T1DM. Though much of the knowledge regarding MSCs has been generated from the use of bone marrow-derived MSCs, studies have also made use of MSCs derived from different tissues including muscle, adipose tissue and umbilical cord blood (da Silva Meirelles, 2006).

Figure 1.12 depicted the immunomodulatory effects of MSCs. The immunomodulatory properties were initially discovered in T cell proliferation assays, in which MSCs were reported to suppress T cell proliferation irrespective of donor source (Bartholomew et al., 2002; Le Blanc et al, 2003). MSCs can significantly suppress the proliferation of both $CD4^+$ and $CD8^+$ lymphocytes, reduce the expression of activation markers on PHA-stimulated lymphocytes and abrogate the

response of memory T-cells to their antigen (Krampera et al., 2003). Additional studies using a transwell culture system to separate leukocytes from MSCs demonstrated an inhibitory effect on leukocyte proliferation suggesting the presence of soluble factors from MSCs for their suppressive properties (Nauta & Fibbe, 2007). Among the many possible candidates representing MSCs-derived suppressive molecules, the transforming growth factor-β (TGF-β), hepatic growth factors (HGF), prostaglandin (E2), and IL-10 have been found to suppress T-cell-mediated antigen responses *in vitro* (Tyndall et al., 2007). Studies showed that the expression of nitric oxide synthase and heme oxygenase-1 in MSCs are responsible for their immunosuppressive properties (Sato et al., 2007; Chabannes et al., 2007). MSCs may also regulate immune response through the interaction of DCs and NK cells (Abdi et al., 2008). Neither of these mechanisms in governing the immuno-modulatory properties of MSCs is exclusive, and their relative contribution may vary in different experimental models.

Due to the lack of expression of MHC class II and most co-stimulatory molecules, MSCs has once been regarded as hypo-immunogenic cells. However, recent studies suggest that MSCs may not be as immuno-privileged as once regarded (Nauta et al,

2006). MSCs indeed express MHC-I and numerous adhesion molecules such as VCAM and ICAM-1. Though they are negative for MHC-II expression, IFN-γ-stimulated MSCs were found to express MHC-II (Le Blanc & Ringden, 2007). It is suggestive that MSCs may only escape from the immune rejection for a short period (Chamberlain et al., 2007).

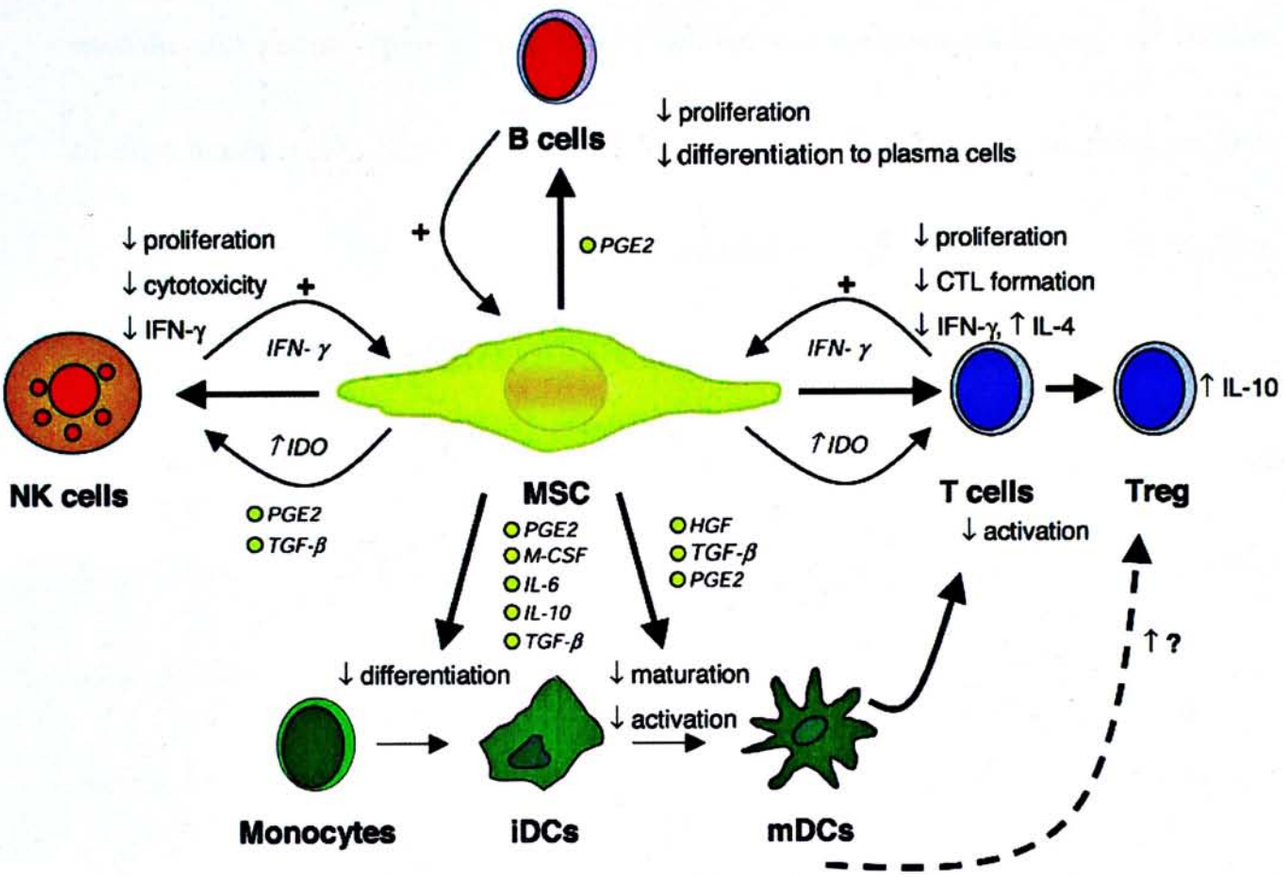

Figure 1.12 Immunomodulatory effects of MSCs.

CTL indicates cytotoxic T cell; HGF, hepatocyte growth factor; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; and TGF-β, transforming growth factor β. *[Extracted from: Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 110(10):3499-506. 2007]*

1.6.3 Neural stem cells

The isolation and culture of neural precursor cells (NPCs) offer a new treatment modality for neural repair, of which they can act as a replacement therapy for the lost of host neural cells. Similarly, the immunogenic status of the donor cells and thus whether they will be rejected after transplantation are of great concern. Studies showed that in vitro culture of primary neural cells would results in up-regulation of MHC molecules (Grenier et al., 1989). A study reported a low expression MHC on neural cells derived from rodent striatum, however the MHC expression increased after differentiation with cytokine exposure (McLaren et al., 2001). It was later reported that only the MHC class I and II molecules, but not the costimulatory molecules, CD40, CD80 and CD86, increase in expression upon culture expansion (Odeberg et al., 2005). PBMCs were unresponsive when co-cultured with these vitro-expanded NSCs, hence suggesting a low immunogenicity of NSCs despite MHC incompatibility and high MHC expression.

1.6.4 Fetal stem cells

The use of fetal stem cells has been extensively utilized in the harvest of umbilical cord blood haemopoietic stem cells (HSCs) and fetal neural tissue for therapeutic use. Of great interest is the potentially accessible source of fetal stem cells from the remaining fetal organs. Fetal stem cells are described as multipotent for their partially determined identity in which the organ they reside in. Though their potential are restricted than the pluripotent ES cells, fetal tissues possess distinctive biological properties that are ideal for transplantation. They have potential in regenerating damaged tissues and are capable of rapid cell growth, proliferation and differentiation. Fetal stem cells also demonstrated considerable reduced immunogenicity to evoke immunogenic responses. Recent report showed that fetal MSC did not elicit alloreactive T-cell proliferation and were able to suppress mitogen induced lymphocyte proliferation (Gotherstrom et al., 2004). Apparently, fetal MSCs possess all the immunomodulatory properties of adult MSCs and what makes them outshine adult MSCs is the greater potent in proliferation and differentiation (Gotherstrom et al., 2004).

1.6.5 Potential immuno-study in human fetal pancreatic stem cells

As mentioned in previous section, human fetal tissues hold a great potential in therapeutic application due to its potent regenerative nature and its likely immune-privileged status. Previous trials employed fetal intestine and fetal kidney for transplantation (Dekel et al., 1997; Lopes et al., 2000). It was noted that in vivo growth and maturation of first-trimester human fetal pancreas obtained at 6-9 weeks of gestation, resulted in normalized glycemia of diabetic mice post-transplant (Castaing et al., 2001). Further studies revealed that the immunogenicity of first-trimester human fetal pancreatic grafts is less than that of second-trimester (Brands et al., 2008). There was a reduced expression of immune-related genes and less cellular infiltration after transplant of first-trimester fetal pancreas. Recently, our laboratory have successfully isolated, cultured and characterized a population of pancreatic progenitor cells (PPCs) derived from human fetal pancreas that are amenable to growth and differentiation into insulin-producing islet-like-cell clusters (ICCs). Nevertheless, their immunological nature has yet to be characterized. It is necessary to characterize the immuno-properties of PPCs, with a particular focus on the immunologic make up of PPCs derived from the first and second trimester.

1.7 Aims and Objectives of study

The procurement of fetal stem cells as a transplantable source for treating T1DM is of increasing interest owing to their higher proliferation and differentiation capacity as well as the immune-privileged status. Several studies have demonstrated the advantages, in terms of reduced immunogenicity, of using fetal tissues from first trimester over second for transplantation.

It is hypothesized that human fetal PPCs and ICCs derived from first-trimester possess lower immunogenicity than that from second-trimester. The aim of this study is to evaluate the immunogenicity of human fetal PPCs and PPC-dereived ICCs. Other specific objectives are:

1. To characterize the expression of some selected immune-related genes in PPCs and ICCs;
2. To examine the effect of interferon-γ on the expression on MHC molecules in PPCs and ICCs;
3. To compare the *in vitro* immunogenicity of PPCs and ICCs from 1st and 2nd trimester;
4. To further compare the immune rejection of ICCs from 1st and 2nd trimester using a xenotransplantation model.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of pancreatic progenitors from human fetal pancreas and induction of Islet-like Cell Clusters differentiation

2.1.1 Tissue procurement

Human fetal pancreas specimens used in this study were procured from aborted fetuses after surgical termination of pregnancy (STOP); they were provided and processed by the Department of Obstetrics and Gynecology, The Prince of Wales Hospital (PWH), The Chinese University of Hong Kong. Specimens were collected during early gestation between 9 and 15 weeks. Informed consent was obtained before any procedures were performed. The approval for the use of fetal tissue in research was obtained from the Clinical Research Ethics Committee (CREC-2005.461). Tissue samples were collected after the completion of surgical termination of pregnancy (STOP) and examined under a high-power dissecting microscope. Fetal tissues including stomach, pancreas, kidney, liver and intestine were identified and washed with sterilized saline prior to isolation. Pancreata at first trimester (9-11week) and second trimester (12-15week) were collected and used in the study.

2.1.2 Tissue processing and PPC culture

The in vitro PPCs induction and ICCs differentiation are schematically

illustrated in Figure 2.1. Briefly, PPCs were grown from fetal pancreatic cell clusters isolated from fetal pancreas as previously described (Suen et al., 2008; Leung et al., 2009). Dissected fetal pancreas was washed with sterilized PBS. They were then minced and digested in HBSS containing 3mg/ml Collagenase P (Roach Molecular Biochemicals, Mannbeim, Germany). The vials were shaken vigorously at 37°C for 10-15 min. The collagenase digestion was terminated by the addition of GIBCO™ Hanks' Balanced Salt Solution (HBSS; Gibco Life Technologies Ltd., Paisley) followed by centrifugation for 5 min at 300 g. The digest was further washed twice by HBSS. The remaining centrifuged pellet containing pancreatic cell clusters were resuspended in 60mm non-treated petri dishes (Corning Incorporated, NY, USA) containing culture medium. The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 20mM HEPES (Invitrogen Life Technologies, CA, USA), 1mM sodium pyruvate, 1% penicillin/streptomycin (Gibco Life Technologies) and 71.5uM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Rounded, non adherent cell-clusters formed within 48h of incubation and were transferred to a new culture dish and incubated for another 48h to deplete adhering fibroblast. After 96h, medium was replaced with modified RPMI 1640 media

supplemented with 20ng/ml each of basic fibroblast growth factor (bFGF) (Sigma-Aldrich) and epidermal growth factor (EGF) (Invitrogen) to induce PPC outgrowth. Within several days cells migrated out from the cell-clusters forming a monolayer of cells. Upon reaching confluence, monolayers were incubated with 0.05% trypsin-EDTA (Invitrogen) for 6 min and the reaction was stopped by Phosphate Buffer Saline (PBS) and the cell suspension was centrifuged for 5 min at 1,600 x g. The cell pellets were further washed twice by PBS. Cell pellets were then resuspended in the modified RPMI 1640 media supplemented with bFGF and EGF and were replated in a new filter-capped T75 cell culture flask (TPP., Trasadingen, Switzerland). PPCs were maintained up to 10 passages and only passages 3-9 were used for experiments in this study.

2.1.3 *In vitro* differentiation of PPCs into islet-like cell clusters

Differentiation of PPCs into islet-like cell clusters (ICCs) were performed as previously reported (Zulewski et al., 2001; Suen et al., 2008; Leung et al., 2009). To initiate ICC differentiation, four T75 cell culture flasks (TPP) of confluent PPCs were required. PPCs were trypsinized at described above and after washing twice with PBS, cell pellets were resuspended in serum-free DMEM/F12 medium containing B27, 0.05% BSA and cocktail of growth factors including 10nM extendin-4 (Sigma), 10 ng/ml HGF (R&D Systems), 500pM betacellulin (Sigma) and 10 mM nicotinamide (Sigma) in an 6-well ultra-low attachment plate (Corning Incorporated) for 8 days. The media was replaced every other day. On day 8, differentiated ICCs were handpicked by micro-pipettes under an inverted light microscope (Leica Microsystems, Wetzlar, Germany) for further experiment. For cytokine treatment, medium in the ICCs culture were replaced by modified RPMI 1640 medium for further incubation.

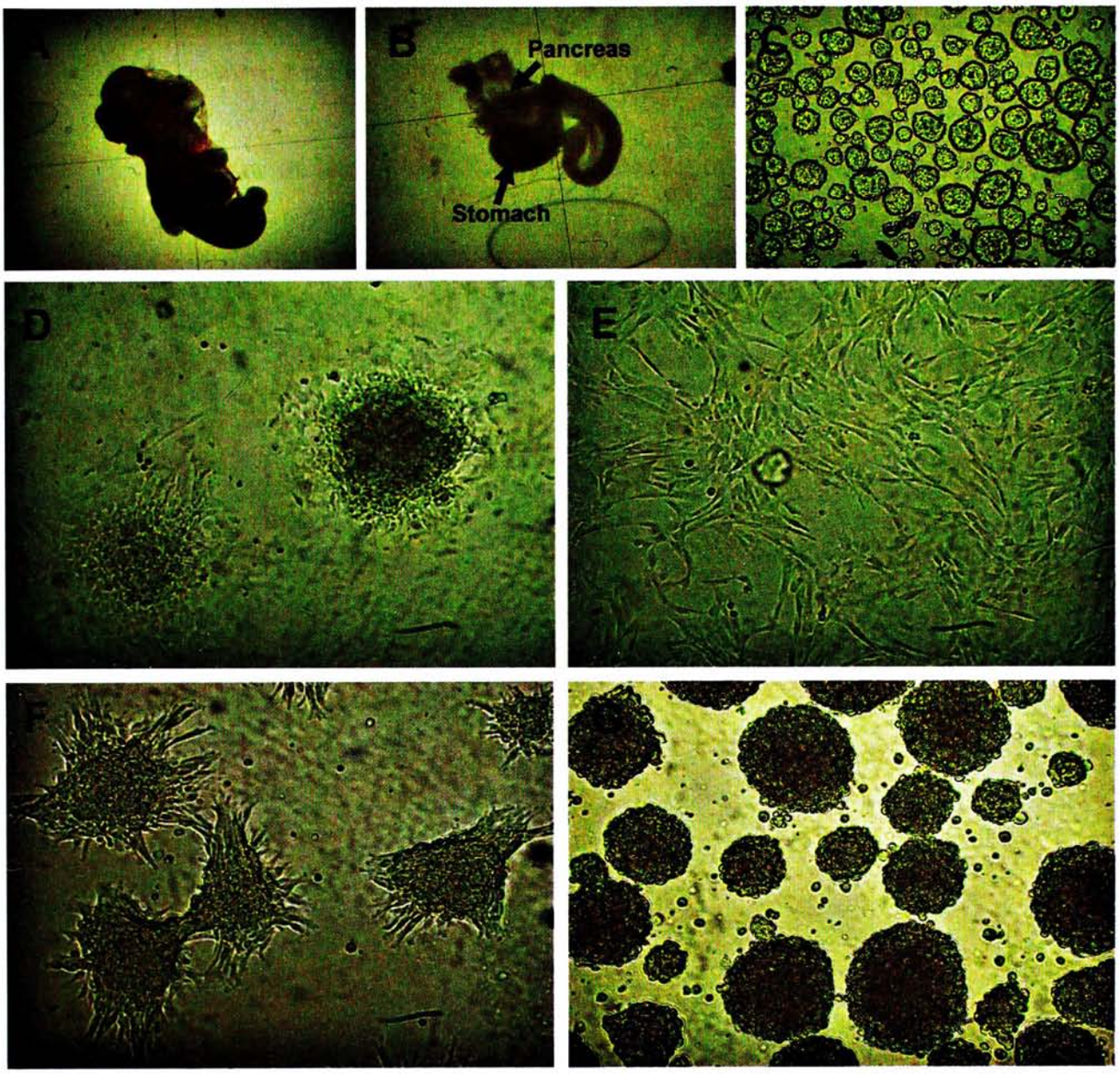

Figure 2.1 Representative microscopic images showing the in vitro PPCs induction and ICCs differentiation. (A) A fetus is collected from Surgical Termination of Pragnancy (STOP); (B) The Stomach is identified, fetal pancreas is usually attached at the back of the stomach; (C) After digestion the fetal islet aggregates are obtained and they will round up within 2 days in the presence of modified RPMI 1640 medium supplemented with 10% FBS; (D) PPC outgrowth from these aggregates is induced in the presence of EGF and bFGF; (E) Monolayer of cells called PPCs are formed within several days; (F) Differentiation is initiated by serum-free DMEM/F12 conditioned medium supplemented with cocktail of growth factors. PPC starts to aggregate within the first 24 hours; (G) After 8 days of differentiation rounded islet-like cell clusters are formed.

2.1.4 Interferon-γ and IL-10 treatment

PPCs were seeded at a concentration of 1×10^5 cells/well in a 6-well plate and waited for at least 4 hours for the attachment of PPCs. IFN-γ (R&D Systems, Minneapolis, MN, USA) of concentrations (100U/ml, 200U/ml and 500U/ml) were added to the PPCs and were incubated for 2 days or 4 days. IL-10 (Peprotech Inc., Rocky Hill, NJ) of concentrations (5U/ml, 25U/ml and 100U/ml) were added to the PPCs and incubated for 2 days. Medium of differentiated ICCs were replaced by modified RPMI 1640 medium prior to IFN-γ and IL-10 treatment.

2.2 Cell culture of human placental choriocarcinoma JEG-3 cell line

JEG-3 cell line was kindly provided by Dr. HF Kung (Stanley Ho Centre for Emerging Infectious Disease, CUHK). JEG-3 cell line was cultured in DMEM medium (Gibco Life Technologies) containing 4500mg glucose, L-glutamine and 110 mg/L sodium pyruvate supplemented with 10% FBS and 1% penicillin/streptomycin. Upon reaching confluence, the monolayer was digested with 0.25% trypsin-EDTA (Invitrogen) for 10 min and the reaction was stopped by PBS. The cell suspension was centrifuged for 5 min at 300 g and was replated onto a new T75 cell culture flask (TPP).

2.3 RNA expression analysis

2.3.1 RNA isolation

Total RNA was isolated from cultured PPCs, ICCs, JEG-3, fetal pancreas and fetal kidney in the presence of 1 ml TRIzol® (Invitrogen) according to manufacturer's instructions with minor modifications. After vigorous vortex for cells and homogenization for fetal tissues, samples were incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Chloroform (0.2ml/ 1ml Trizol) was added to each sample. The mixture was shaken vigorously for 15 s and allowed to incubate for 3 additional minutes at room temperature. The vials were then centrifuged at 12,000 g for 15 min at 4°C. After centrifugation, the upper RNA-containing aqueous phase was carefully collected and precipitated with isopropyl alcohol (1ml/ 1ml Trizol®) and glycerol (4-8 ul/ 1 ml Trizol®) at -80°C overnight. The next day samples were centrifuged at 12,000 g for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 1ml ice-cold 75% ethanol, which was further centrifuged at 7,500 g for 15 min. Finally pellets were air dried for 15 min and dissolved in 15-20 µl RNAase-free deionized water (Invitrogen). The aqueous RNA was incubated at 58°C for 5 min to dissolve RNA before chilling on ice.

The amount of total RNA was quantified using a UV-spectrophotometer. Concentration of RNA was calculated from the value of absorbance at wavelength 260nm (O.D. 260) x 40 x dilution factor. The purity of RNA obtained was determined by comparing the ratio of O.D. 280 to O.D. 260 and the value fell between 1.6 and 2.0 was considered as pure (Leung et al., 2009). The integrity and quality of RNA were checked by RT-PCR for the house-keeping gene β-actin.

2.3.2 Reverse transcriptase (RT)

First strand cDNA was reverse transcribed using Superscript™ III Reverse transcriptase (Invitrogen) according to the manufacturer's protocol. In brief, 2-3 μg RNA was combined with 500 μg Oligo(dT)188, 10mM dNTP mix to make up a 13 μl reaction mix. This reaction mix was denatured at 65°C for 5 min. After quick chill on ice for 1 min, 5X first-strand buffer, 0.1M DTT, 40 units/μl RNAaseOUT™ and 200 units/μl of Superscript™ III Reverse Transcriptase were added to yield 20μl 1X reaction mixture. The reaction was incubated for 1 h at 50°C and was finally terminated by incubating at 70°C for 15 min. The resulting first-strand cDNA was directly subjected to conventional polymerase chain reaction (PCR) or real-time PCR amplification for the investigation of

specific gene expression.

2.3.3 Design of primers for PCR and real-time PCR

PCR primers for different genes were designed from human cDNA sequence using online software Primer 3 version 0.4.0 (Rozen and Skaletsky, 2000). Procedures were done according to the description by the software. Briefly, primers for each gene target were selected containing minimal internal structure (i.e. hairpins and primer-dimers formation as determined by the software) and having compatible primer size (Optimum: 20 $\pm$3 b.p. of each complimentary pairs), optimal melting temperature (Optimum: 60 $\pm$3^{o}C of the other), and optimal GC content (Optimum: 50 $\pm$20% of the other). Product size ranging from 50-200 base-pairs (b.p.) of each target gene was picked. Details of the sequence of the primers are shownwere depicted in Table 2.1. Each primer was tested by conventional PCR (procedure described in section 2.3.4) using positive cDNA as template. Only those primers which generate a single band of expected product size were used for further experiment. Besides, a cDNA dilution curve with at least 10 serial 2-fold dilutions was obtained from appropriate real-time PCR experiments (Procedures described in 2.3.5) and the PCR efficiency for each primer was evaluated.

Gene	Accession No.	Primer sequence	Location (nucleotides)	Expected size (bp)
MHC-II (HLA-DRA)	NM_019111.3	5'-GGACAAAGCCAACCTGGAAA-3'(sense)	351-370	120
		5'-AGGACGTTGGGCTCTCTCAG-3'(antisense)	470-451	
CD80	NM_005191.3	5'-AGGGAACATCACCATCCAAG-3'(sense)	235-254	181
		5'-TGCCAGTAGATGCGAGTTTG-3'(antisense)	415-434	
CD86	NM_006889.3	5'-AGCTTTGCTTCTCTGCTGCT-3' (sense)	22-41	142
		5'-ATCCAAGGAATGTGGTCTGG-3'(antisense)	163-182	
HLA-G	NM_002127	5'-CCTGGACTCACACGGAAACT-3'(sense)	29-48	64
		5'-AACCTGTCACCTAATGGGAGTG-3'(antisense)	92-113	
B7H4	NM_024626.2	5'-CAGATGCTGGCACCTACAAA-3' (sense)	444-463	130
		5'-AAGGTCTCTGAGCTGGCATT-3'(antisense)	573-592	

Table 2.1 Primers-pairs designed and used for the study of immuno-related gene expression in PPCs and ICCs

2.3.4 Polymerase chain reaction (PCR)

The first strand cDNA was subjected to PCR reaction. Oligonucleotide primers and expected PCR product sizes which were cited in the literature are summarized in Table 2.2 (Invitrogen). PCR was performed in 25µl reaction volume containing 5 µl template cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.3 µM each of sense and anti-sense primer, PCR buffer and *Taq* DNA polymerase (Invitrogen). The reaction was initiated at 94°C for 3 min for denaturation, then annealing at temperature ranging from 60-64°C for the gene of interest, and finally extending at 72°C for 30 s. PCR cycles were repeated for 34 cycles. PCR products were then electrophoresed on a 2% agarose gel (wt/vol; Cambrex Corporation, Charles City, IA, USA) in 1X Tris-Acetate-EDTA (TAE) buffer pre-stained with ethidium bromide for 15 min at 100V and the resulting bands were visualized using a camera equipped with an UV illuminator (FluroChem 8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging, Alpha Innotech Corporation, CA, USA)

Gene	Primer sequence	Expected size (bp)	Reference
HLA-A	5'-TGTCCCTCACAGCTTGTAAAG-3'(sense) 5'-ATTATGCCTACACGAACACAG-3'(antisense)	150	Brands et al., 2008
CCL-19	5'-AGCAGTTAACCTATGACCGTGC-3'(sense) 5'-CCAGGCGGCTTTATTGGTAGC-3'(antisense)	245	
C3	5'-ACGAATGCCAAGACGAAGAG-3'(sense) 5'-CTGAAGCTTTATCTGGAGTGGG-3'(antisense)	126	
TNFSF10	5'-GATCAAGACCATAGTGACCAA-3'(sense) 5'-TGGCATGATCTCACCACAC-3'(antisense)	167	
β-actin	5'-TGGCACCACACCTTCTACAATGAGC-3'(sense) 5'-GCACAGCTTCTCCTTAATGTCACGC-3'(antisense)	396	Huang et al., 2003
β-actin (Real-time)	5'-TGTCCACCTTCCAGCAGATGT-3'(sense) 5'-CGGACTCGTCATACTCCTGCTT-3'(antisense)	51	Suen et al., 2006

Table 2.2 Primer-pairs used for the study of immuno-related gene expression in PPCs and ICCs

2.3.5 Real-time PCR analysis

Real-time PCR were performed using i-Cycler Thermal Cycler (version 3.1; Bio-Rad Laboratories Incorporation, Munich, Germany) as previously described (Leung et al., 2009), with the following conditions: 40 cycles of 94°C for 15s, 60-62°C for 1 min depending on the gene of interest, and 72°C for 30s. Reactions were performed in triplicate, in 25 µl, with 5µl 5-fold diluted cDNA, SYBR Green PCR Master mix (Bio-Rad Laboratories Incorporation) and 0.3 µM each primers (Invitrogen). Melting curve (+ 0.5°C for every 10 s) analysis was performed to confirm amplification specificity of the PCR products. Amplification data was collected using an i-Cycler Detector and analyzed using the Sequence Detection System Software. Results are normalized to β-actin and the relative mRNA level of the gene of interest is expressed as $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001). The schematic diagram of real-time PCR using SYBR Green assay is shown in Figure 2.2.

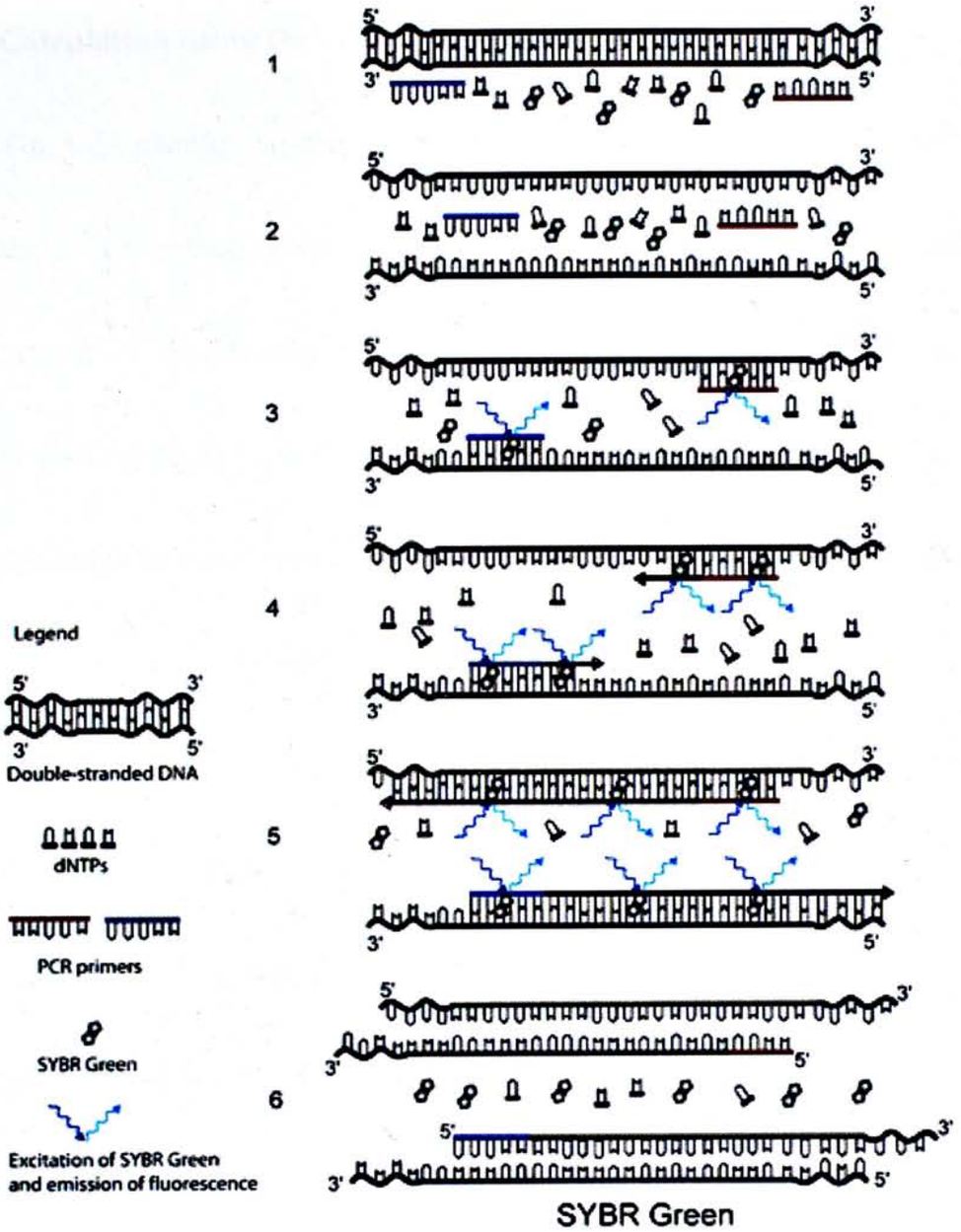

Figure 2.2 The principle of SYBR Green detection in real-time PCR. (1-2) The fluorescent dye SYBR Green is added to the mixture of PCR. SYBR Green is a DNA dye that bounds only to double-stranded DNA and it emits fluorescence upon excitation. (3) At the beginning of the reaction light emission is low since little double-stranded DNA is present. (4) As the reaction proceeds, the amount of double-stranded DNA increases and hence emission of fluorescence increases. (5)C_T value is calculated based on the time at which fluorescent emission increases beyond a threshold level. (6) The denaturation steps contain only single-stranded DNA and signal is only detected during annealing and extension. [*Picture extracted from: Diagnostic Procedures/ Stool Specimens/ Molecular diagnosis "http://www.dpd.cdc.gov/dpdx/html/frames/diagnostic procedures/body_dp_stool_rt pcr_illust.htm" on 07June2010*]

2.3.6 Calculation using the comparative C_T method

The fold changes in the target genes mRNA expression were calculated using the $2^{-\Delta\Delta C_T}$ method (Suen et al., 2008). SYBR Green is a dye that binds the minor groove of double-stranded DNA and emits fluorescence upon excitation. In Real-time PCR, C_T value is calculated based on the time at which fluorescent emission increases beyond a threshold level. The fluorescent intensity correlates to the level of target mRNA; a greater quantity of input mRNA results in a lower C_T value since less PCR cycles are required for the fluorescent emission beyond the threshold. The ΔC_T value of a target gene is calculated by subtracting the C_T derived from the house-keeping gnen β-actin. The $\Delta\Delta C_T$ value is the differential gene expression of two samples which is calculated by subtraction of the control ΔC_T from ΔC_T of according samples. The expression level of the respective target gene relative to the control ΔC_T is calculated using the equation $2^{-\Delta\Delta C_T}$.

2.4 Flow cytometry

The basic principle of flow cytometry is illustrated in Figure 2.3. PPC and ICCs treated with PBS control or 200U/ml IFN-γ for 48 hr were harvested and collected by centrifugation. ICCs were segregated into single cells by using 0.05% trypsin (Invitrogen) for 15 mins followed by gentle pipetting. Cells at concentration 1×10^7 cell/ml were resuspended in ice-cold staining buffer (1X PBS supplemented with 1% FBS). Suspended cells were blocked with normal donkey serum in ice-cold staining buffer on ice for 30 min. They were then incubated with 20 μl FITC-conjugated mouse anti-Human HLA-A,B,C antibody (BD Biosciences) on ice for 30 min. Cells were then washed by cold staining buffer for three times and reconstituted in 700μl cold staining buffer for cytometric analysis. Isotype control samples were incubated with 20 μl FITC-conjugated mouse IgG1κ isotype antibody (BD Biosciences). Cytometric analysis was performed by a CytomicsTM FC500 flow cytometer (Beckman Coulter, Fullerton, CA).

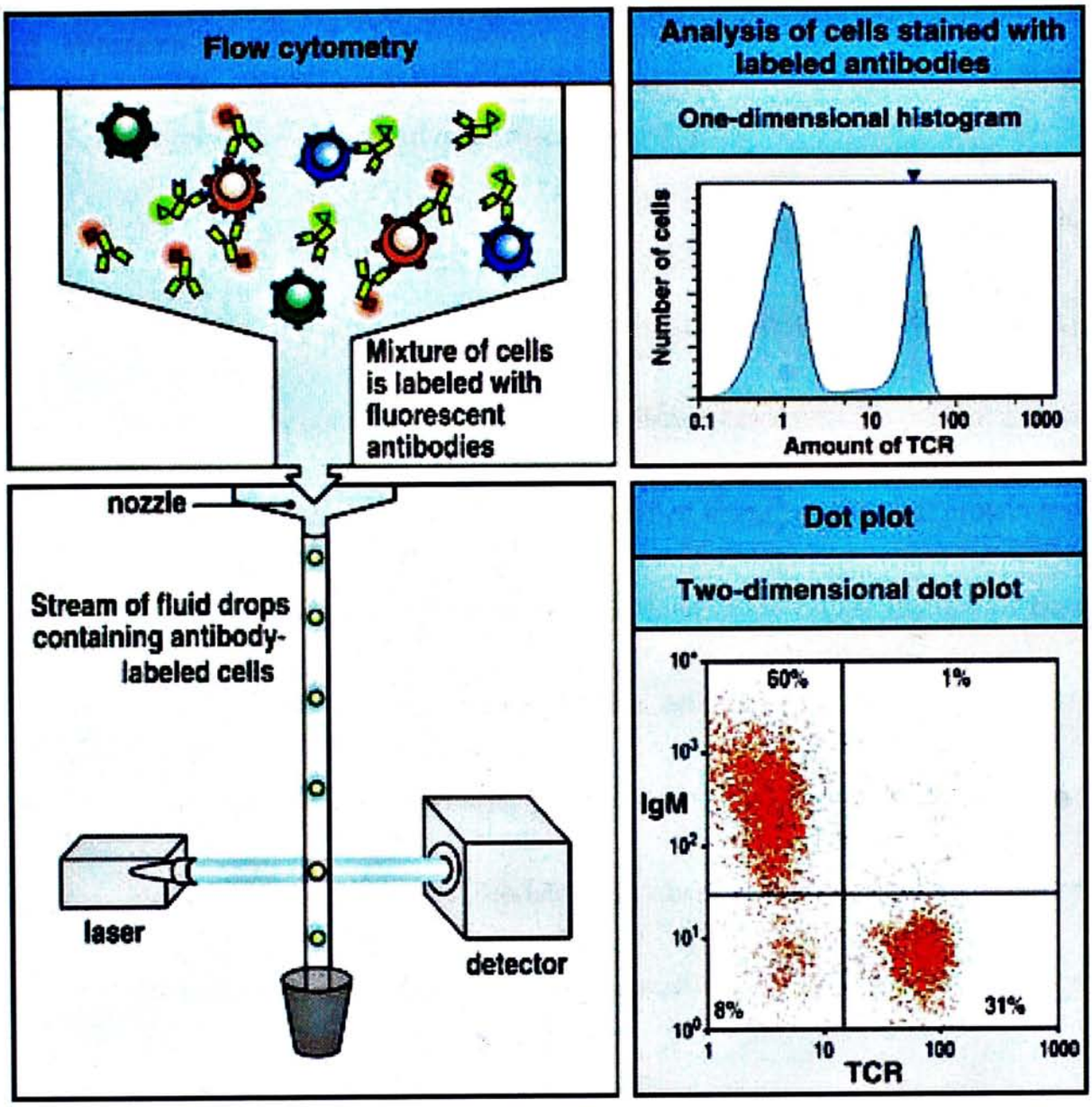

Figure 2.3 The basic principle of flow cytometry and two classical data output formats. (Left-hand side) Cells are labeled with one or more types of fluorescent antibodies. The sample in sheath fluid is hydrodynamically focused to pass through a laser-detector system in which the fluorescent character of each cell is measured. (Right-top) One dimensional histogram showed a single parameter of fluorescence on the x-axis and the number of events (cell count) on y-axis. (Right bottom) Two dimensional dot plot displays two measurement parameters, one on x-axis and one on y-axis, while the cell count is shown as a density plot. The parameters can be relative fluorescence or light scatter intensity. *[Extracted and modified from http://www.microvet.arizona.edu/courses/VSC519/Secure/CaseNoIg/Case4NoIg.html on 7June2010]*

2.5 Western blot analysis

2.5.1 Protein extraction and quantification

Cell lysates from PPCs and JEG-3 were prepared using Cytobuster Protein Extraction Reagent (Novagen, Darmstadt, Germany). The mixtures were incubated at room temperature for 15 min and centrifuged at 16,000 g at 4°C for 15 min. Protein concentration was quantified using the Bio-Rad protein assay kit (Bio-Rad Laboratories Incorporation), which based on the method of Bradford. Protein samples were incubated with an acidic dye for 8 min at room temperature before measuring the absorbance at 595 nm with a spectrometer (Version 1.3, KCjunior, Bio-tek Instrument Inc., VT, USA). The relative determination of protein concentration was interpolated with the 0.1-0.5 mg/ml BSA standard curve.

2.5.2 Western blotting

Western blotting procedures were performed as previously reported with minor modifications (Leung et al., 2009). Briefly, protein samples were mixed with Laemmli buffer containing 5% β-mercaptoethanol. They were heat-denatured at 100°C for 5 min. Denatured protein samples (15-30 μg/lane) were subjected to

electrophoresis on a 12% SDS-polyacrylaminde gel at 100V for 2 h. Proteins were then immobilized on 0.45 micron polyvinylidene difluoride (PVDF) transfer membrane (GE Osmonics Labstore, Minnetonka, MN, USA). Electroblotting was conducted by Semi-Dry Transfer System (Bio-Rad Laboratories Incorporation) at 17 volts for 1 h. The blotted protein was blocked in 5% (wt/vol) of skimmed milk in PBS and 0.1% (vol/vol) of Tween 20 for 1 h at room temperature. The membrane was subsequently incubated in primary antibodies including mouse anti-HLA-G (1:250; EBioscience, San Diego, Calif., USA) and mouse anti-β-actin (1:8000; Abcam, Cambridge, MA) overnight at 4°C, followed by peroidase-labelled sheep anti-mouse IgG antibody (1:2500; Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature. All antibodies were diluted in 1% BSA (wt/vol) supplemented with 0.5% (vol/vol) Tween-20 (Sigma-Aldrich). After thorough washing, positive bands were visualized using enhanced chemiluminescent (ECL) plus Western blot detection reagents and autoradigraphy film (Amersham Biosciences). The chemiluminescent intensity of the protein bands was quantified using an image analyzer (Molecular Dynamic Imaging Quant, Sunnyvale, CA, USA).

2.6 Mixed lymphocyte reaction (MLR)

2.6.1 Isolation of peripheral blood mononuclear cells (PBMC)

Human fresh blood or DMSO frozen blood samples were kindly provided by Dr. K.S. Tsang (Department of Anatomical and Cellular Pathology, CUHK). PBMCs isolation procedures were performed as previously reported with minor modifications (Boyum 1968; Bain & Pyshyk, 1972; Jin et al., 1987) The PBMCs isolation process was depicted in figure 2.4. Blood samples were diluted at a ratio of 1:20 by ice-cold PBS supplemented with 10% FBS. The vials were then centrifuged at 400 g for 10 min at room temperature. For frozen blood samples the pellet was washed once by ice-cold 10% FBS/PBS to remove DMSO. Cell pellets were then resuspended in 10% FBS/PBS. The cell suspension was overlaid on Ficoll-Paque^{TM} Premium (Amersham Biosciences) of specific density of 1.077 and PBMCs can be isolated by centrifugating at 400 g for 30 min at room temperature with brake off. The upper plasma layer was removed till 5 mm above the interface of Ficoll Hypaque portion and PBMCs were collected at mononuclear cell layer at the plasma-ficoll interface. A volume of the density gradient until 5 mm above red blood cell layer were also harvested to increase the yield. The yield was washed twice with ice-cold 10% FBS/PBS to

remove ficoll. The cell pellet was resuspended with modified RPMI 1640 medium and the cell number was adjusted to 2×10^7 cell/ml in a T75 cell culture flask. The cells were incubated at 37°C in a humidified 5%-CO_2-incubator for 1-2 h to allow the adherence of monocytes to culture surface. The flask was rocked gently and monocyte-depleted lymphocytes were aspirated to another T75 flask. Viable lymphocytes were cultured for a couple of days. Cell viability was confirmed under light microscope by 0.5% trypan blue, based on the rationale that viable cells were able to exclude the dye whereas non-viable cells were stained blue. Cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Total number of unstained cells counted}}{\text{Total number of cells counted}} \times 100\%$$

Only samples with the viability over 90% would be used in this study.

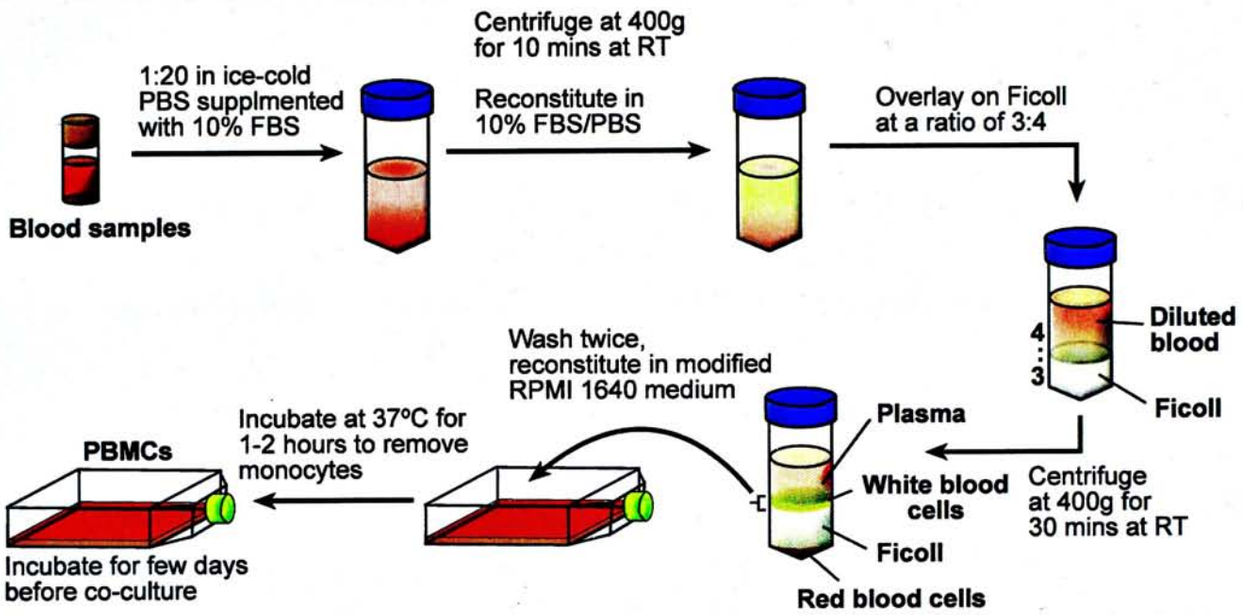

Figure 2.4 Schematic diagram of human peripheral blood mononuclear cells (PBMCs) isolation by ficoll gradient. Diluted blood is overlaid on Ficoll of specific density of 1.077 and PBMCs can be isolated by centrifugating at 400 g for 30 min at room temperature with brake off. PBMCs were collected at mononuclear cell layer at the plasma-ficoll interface. Red blood cells are at the bottom of the tube. The yield was washed twice with ice-cold 10% FBS/PBS to remove ficoll. Cell pellet was resuspended with modified RPMI 1640 medium in a T75 cell culture flask. The cells were incubated for 1-2 h to allow the adherence of monocytes to culture surface. The flask was rocked gently and monocyte-depleted lymphocytes were aspirated to another T75 flask.

2.6.2 PPC-PBMC MLR

Cell number-dependent unidirectional (one-way) MLR were performed by co-culturing isolated PBMCs (2×10^5 cells/well) with irradiated PPCs (1500 rad) at different cell numbers (1×10^2, 1×10^3, 1×10^4, 5×10^4, 1×10^5 cells/well) in a 96-well plate (Corning Incorporated). MLR for comparing first- and second-trimester PPCs were performed by co-culturing PBMCs (2×10^5 cells/well) with irradiated PPCs (5×10^4 cells/well). Allogenic control was performed by co-culturing PBMCs with mismatched irradiated PBMCs (3000 rad) as previously reported Chong et al., 1990), of each 2×10^5 cells/well. For all experimental setup the modified RPMI 1640 medium for PPCs as described in section 2.12 was used in MLR. The MLR was cultured for 6 days for proliferation assay (refer to Section 2.5.4) or 3 days for cytokine assay (refer to Section 2.8).

2.6.3 ICC-PBMC MLR

MLR were performed by co-culturing isolated PBMCs ($2X10^5$ cells/well) with approximately 50 irradiated ICCs (1500 rad) in ultralow 24-well plate (Corning Incorporated). The modified RPMI 1640 medium for ICCs were used in MLR.

The MLR was cultured for 6 days for proliferation assay (refer to Section 2.5.4).

2.6.4 Proliferation assay

Cell proliferation Biotrak™ ELISA System (version 2; GE Healthcare, Orasy, France) was employed to assay PBMC proliferation. Cell proliferation was evaluated based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferating cells. The technique makes use of the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. BrdU is detected by immunoassay. The procedures were performed according to manufacturer's instructions. Briefly, 18 h before the PPCs/ICCs and PBMCs have been co-cultured for 6 days, 10mM BrdU labeling solution were added to the culture and incubated at 37°C. For PPC-PBMC MLR, medium with the labeling solution were removed on day 6. For ICC-PBMC MLR, ICCs were filtered from the medium through a nylon net filter of pore size 160um (Millipore Corporation, Bedford, MA) carried by 13mm Swinnex® Filter Holder (Millipore). The remaining filtrate containing medium and PBMCs were plated into a 96-well plate and spun at 400 g in a microplate centrifuge. Plates were air-dried at 60°C for 1 h. The cells were fixed

and then blocked for 30 min each at room temperature. A peroxidase-conjugated mouse monoclonal anti-BrdU antibody was added and incubated for 90 min at room temperature. After three washes, BrdU was visualized by adding the substrate reaction. The absorbance of each well was measured at 450 nm using the spectrometer (Version 1.3, KCjunior).

2.6.2 PPC-PBMC MLR

Cell number dependent unidirectional (one-way) MLR were performed by co-culturing isolated PBMCs (2×10^5 cells/well) with irradiated PPCs (1500 rad) at different cell numbers (1×10^2, 1×10^3, 1×10^4, 5×10^4, 1×10^5 cells/well) in a 96-well plate (Corning Incorporated). MLR for comparing first- and second-trimester PPCs were performed by co-culturing same number of PBMCs with irradiated PPCs cell numbers of 5×10^4 cells/well. Allogenic control was performed by co-culturing PBMCs with mismatched irradiated PBMCs (3000 rad) as previously reported (Chong et al., 1990), of each 2×10^5 cells/well. For all experimental setup the modified RPMI 1640 medium for PPCs as described in 2.12 were used in MLR. The MLR was cultured for 6 days for proliferation assay (refer to section 2.5.4) or 3 days for medium cytokine level assay (refer to Section 2.8).

2.6.3 ICC-PBMC MLR

MLR were performed by co-culturing isolated PBMCs ($2X10^5$ cells/well) with approximately 50 irradiated ICCs (1500 rad) in ultralow 24-well plate (Corning Incorporated). The modified RPMI 1640 medium for were used in MLR. The

2.7.2 Procedures of ICC transplantation

The methodology for ICCs transplantation which has been described previously was used in this study (Suen et al., 2006). The procedures of transplantation are illustrated in Figure 2.5. A total of 22 animals was operated. Briefly, 1,000-1,500 ICCs of similar size derived from the first and second trimester after 8 days of differentiation were picked under the microscope. The differentiation medium was removed from the selected ICCs by centrifugation and ICCs were resuspended in a small volume of sterilized 0.9% (wt/vol) saline. ICCs were then carefully packed into the tip of MICROMAN® positive displacement pipette (Gilson, Middleton, Wisconsin). STZ-treated C57BL/6J mouse was anesthetized with an intraperitoneal injection of 1% (vol/vol) ketamine (Alfasan, Woerden, Holland) and 0.1% (vol/vol) xylaxin (Alfasan). The left abdomen of the mouse was cut opened. The left kidney was exposed and kept moisturized by 0.9% sterilized saline. A small incision was made on the left renal capsule. ICCs were gently introduced between the renal parenchyma and the capsule by using a displacement pipette, followed by suturing of the skin opening. Sham-operated mice were injected with 0.9% sterilized saline. Blood glucose levels of the transplanted mice were monitored for two weeks. Blood was withdrawn from

tail snip and blood glucose level was assessed using a handheld glucometer (Bayer Corporation, Emeryville, CA, USA).

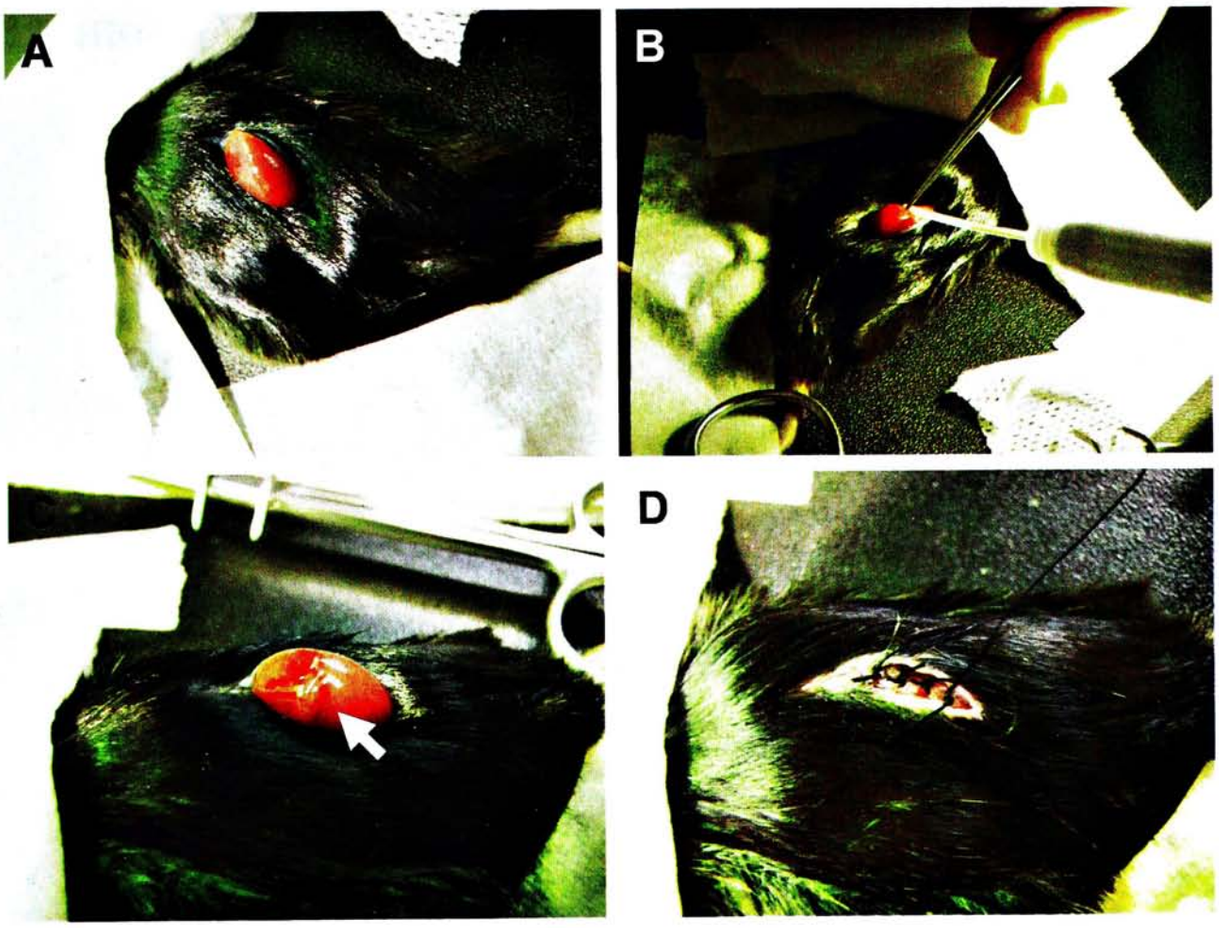

Figure 2.5 Islet-like cell clusters transplantation to C57BL/6J mice. (A) The left abdomen of an anesthetized C57BL/6J mice was cut opened and left kidney was exposed. A small incision was then made on the left kidney capsule. (B) ICCs were gently introduced between the renal parenchyma and the capsule by the displacement pipette. (C) The transplanted ICCs graft was visible within the renal capsule as indicated by the white arrow. (D) The skin incision was sutured at the end of the operation.

2.8 Histological analysis of ICC graft

H&E staining and immunohistochemistry were performed according to our previously reported with some minor modifications (Suen et al., 2006)

2.8.1 H&E staining

Grafted kidneys were fixed in 4% (wt/vol) paraformaldehyde (PFA;Sigma Aldrich) in PBS at 4°C for 48 h. Fixed grafts were washed twice by PBS and then subjected to sucrose gradients as follows: 10% sucrose/PBS for 12h; 20% sucrose/PBS for 24h; 30% sucrose/PBS for 24h. They were then embedded in Tissue-Tek® O.C.T. Compound (Optimal Cutting Temperature Compound; Sakura Finetek, Torrance, CA, USA) and were frozen in -80°C overnight. Embedded kidneys were sectioned at 5-6 μm by LEICA CM1100 Benchtop Cryostat (Leica Microsystems) and were mounted onto silane-coated slides. These slides were air dried at room temperature for few hours and the O.C.T. (Sakura) was removed from the sections by rinsing in PBS twice prior to staining process. Sections were stained with hematoxylin for 5-8 min to visualize the nucleus. The intensity of hematoxylin was optimally adjusted by rinsing with 1% acid alcohol and Scott's tap water. Sections were

counter-stained with 1% eosin for 5 min to visualize the cytoplasm. The slides were de-hydrated by passing through a sequential gradient of ethanol (70% to absolute ethanol, each for 30s) and xylene (three times, each for 30s), and then eventually mounted by the microscopic Entellan® rapid-mounting media (Merck, Germany). Histological changes were examined under a light microscope equipped with a DC200 digital camera (Leica Microsystem).

2.8.2 DAB staining

For the immunostaining of *PDX-1,* sections were incubated with 3% hydrogen peroxide in PBS for 10 min to denature endogenous peroxidase activity and were washed in PBS for three times. They were then permeabilized with 0.1% Tritox-X in PBS for 10 min and washed. Blocking of non-specificity was done by incubating sections with 4% normal donkey serum (Sigma) in 1:20 (vol/vol) Tris Buffered Saline & Tween 20 (TBS-T; Thermo Scientific, Waltham, MA) supplemented with 2% bovine serum albumin (BSA; Invitrogen) at room temperature for 1 h. Rabbit anti-*PDX-1* antibody (Millipore) with dilution 1:200 were applies to slides and incubated at 4°C overnight. They were washed with TBS-T and incubated with donkey anti-rabbit secondary antibody conjugated

with HRP (Amersham Biosciences) at dilution of 1:200 for 1 h at room temperature. Slides were washed and developed with 3,3-diaminobenzidine (DAB; Vector Laboratories, CA, USA) for 5 min. After washing, sections were dehydrated and mounted as described above. The omission of primary antibodies was used as negative controls.

2.8.3 Immunofluorescence staining of CD45

Immunofluorescence staining of CD45 was performed as previously described (Leung et al., 2009). Briefly, samples were blocked with 4% normal donkey serum in TBS-T supplemented with 2% bovine serum albumin (BSA) at room temperature for 1 h. They were then incubated with rat anti-CD45 antibody (1:10; BD Biosciences) overnight at 4 °C. Slides were washed three times with TBS-T and were then incubated with Rhodamine-conjugated goat anti-rat (1:200) secondary antibodies (Abcam) together with 4'6'-Diamidino-2-phenylindole (DAPI; 1:1000; Invitrogen) at room temperature for 1 h. Sections stained with secondary antibody alone were used as negative control. All sections were washed thoroughly with TBS-T, mounted in Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA) and imaged using a

fluorescence microscope and DC 200 digital camera (Leica Microsystem).

2.9 Enzyme-linked immunosorbent assay

The IFN-γ and IL-10 levels in the fresh and spent media were assayed qualitatively with Quantikine® Human IFN-γ ELISA kit and Human IL-10 ELISA kit, respectively (R&D Systems). In brief, irradiated PPCs were seeded at 5×10^4 cells/well in 96-well plates and co-cultured with 2×10^5 PBMCs in the presence of 5mg/ml PHA-P (Sigma). Spent media were collected on day 3 cultures after removal of particulates by centrifugation at 300g. Assays were done immediately. Otherwise, they were aliquoted and stored at -80°C until analysis. Repeated freeze-thaw procedures were avoided for each frozen sample. In accordance with the manufacturers' instructions, the media were diluted using the assay diluent in the 96 well plate coated with polyclonal antibody against IFN-γ or IL-10 and were incubated for 2 h. The mixtures were removed and washed 4 times. IFN-γ– or IL-10-conjugated polyclonal antibodies were added to each well and incubated for 2 h. Substrate solution was added to each well after washing for 4 times and incubating for 30 min in dark. Stop solution was added to each well and the optical density was determined within 30 min using the spectrometer (Version 1.3, KCjunior) at 450 nm, with the wavelength correction at 540 nm. Actual concentrations were obtained from the curve of mean absorbance with

standardized 0-500 pg/ml IFN-γ or IL-10 controls.

2.10 Statistical data analysis

Results were expressed as mean ± SEM for all groups. Multiple comparisons among groups were performed using an ANOVA followed by Tukey's *post hoc* test. When only two groups were compared, probabilities (p value) of chance differences were calculated with Student's unpaired two-tailed t test. Graphical and statistical analyses were conducted using a GraphPad Prism 5 software package (GraphPad Software Inc., San Diego, CA). For all comparisons, $p<0.05$ was considered statistically significant. For real-time RT-PCR, the relative expression was normalized to *β-ACTIN* and calculated using the comparative C_T method, with fold change defined as $2^{-\Delta\Delta C_T}$, as described previously (Lau et al., 2004).

CHAPTER 3

RESULTS

3.1 Immuno-characterization of PPCs and ICCs

To characterized the immunologic nature of our PPCs and ICCs, the mRNA expression of some selective immune-related genes, including HLA-A, MHC-II, complement component 3 (C3), chemokine ligand (CCL19), tumor necrosis factor super family (TNFSF10), innate molecules (B7H4), co-stimulatory molecules, CD80 and CD86, along with non classical MHC-I molecule HLA-G were evaluated by semi-quantitative RT-PCR (Figure 3.1). Results showed that all genes, except for co-stimulatory molecules, CD80 and CD86, were expressed by both PPCs and ICCs. The expression of MHC-I and MHC-II molecules by PPCs and ICCs suggested that they were able to present antigens to host T-cells for activation,thus triggering immune response. The existence of these molecules lends support to the equipped antigen presenting machinery of PPCs and ICCs. Conversely, the absence of co-stimulatory molecules, CD80 and CD86, is indicative of the immuno-privileged status of these cells. Of particular interest is the expression of the non-classical MHC class I molecule HLA-G by both PPCs and ICCs.

To compare the immunogenicity of PPCs with fetal pancreas (Figure 3.2), the mRNA expression of MHC-I derived from these samples were further examined by quantitative real-time PCR. Result showed that the expression of MHC-I on a 12 week fetal pancreas ($2^{-\Delta\Delta CT}$; 2.93±0.225) was 3-fold higher than that of the isolated PPCs of the same week.

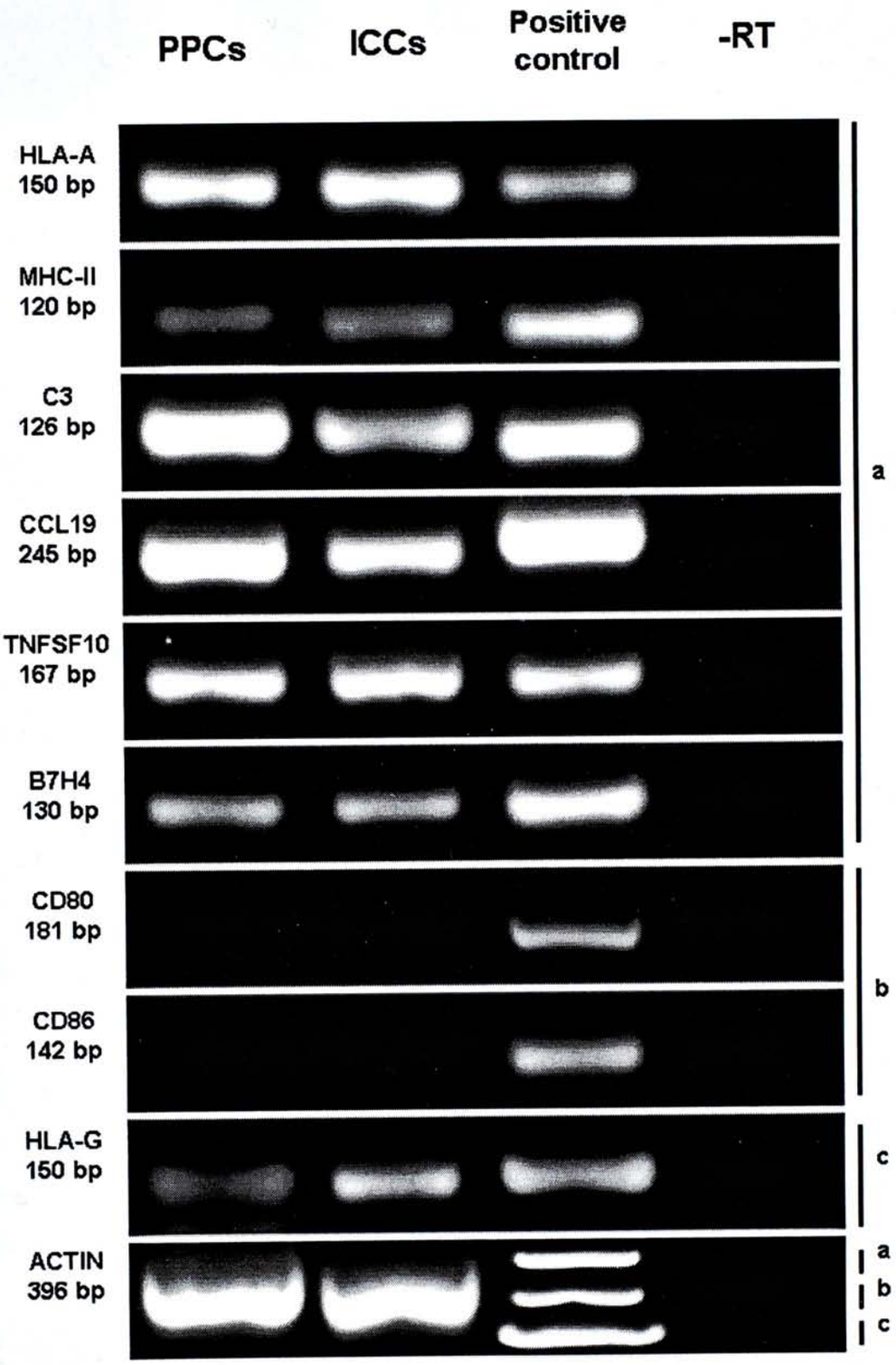

Figure 3.1 The mRNA expression of some selected immune-related genes from PPCs and ICCs. Expression of HLA-A, MHC-II, complement component 3 (C3), chemokine ligand (CCL19), tumor necrosis factor super family (TNFSF10), innate molecules (B7H4), costimulatory molecules CD80 and CD86 as well as HLA-G are evaluated by RT-PCR. Human β-actin is used as an internal control while another three positive controls are employed, i.e. (a) fetal kidney; (b) HSCs; (c) JEG-3.

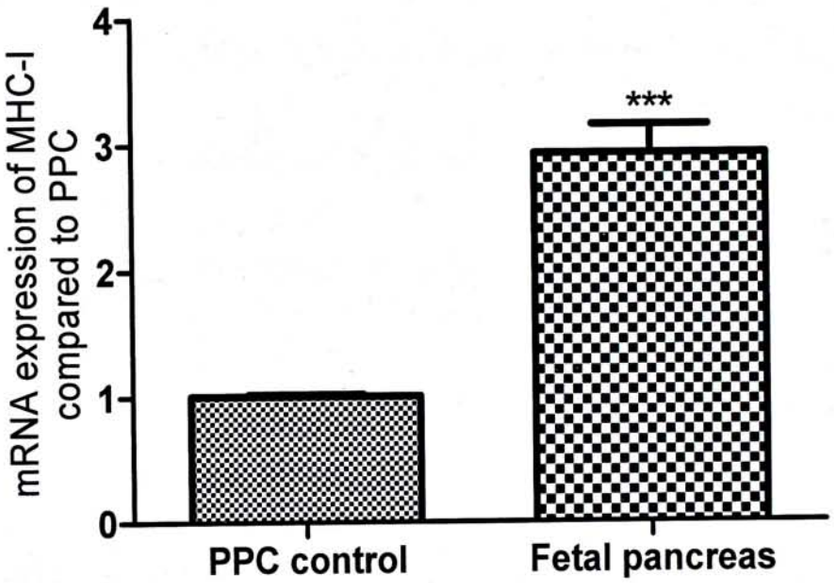

Figure 3.2 Real-time RT-PCR analysis of the mRNA expression of MHC-I molecules from PPCs and human fetal pancreas. MHC-I expression by fetal pancreas is 3-fold higher than that of PPCs. The relative expression is normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ***denotes $p<0.001$ when compared to PPC control.

3.2Effect of cytokines on immuno-properties of PPCs/ICCs

3.2.1 Effect of IFN-γ on MHC-I expression in PPCs

To investigate the effect of IFN-γ on MHC-I expression, PPCs were cultured with 100, 200 and 500U/mL of human recombinant IFN-γ for 2 and 4 days. Quantitative real-time PCR results revealed that the expression of MHC-I molecules in the PPCs was dose-dependently and time-dependently upregulated by IFN-γ (Figure 3.3). In this regard, IFN-γ of dosage 200U/mL for 2 and 4 days (11.08±0.499 vs 14.73±1.45 respectively) induced an increase in MHC-I expression of more than 10 folds, and the fold change accounted for >20 folds when they were cultured with 500U/mL for 4 days (22.27±1.91). In the subsequent experiments we employed 200U/mL IFN-γ for 2 days in the following experiments as widely employed in other studies (Kirchheimer et al., 1988; Ljunggren & Anderson, 1998; Conte et al., 2003; Wen et al., 2004;).

The induction of expressions of MHC-I molecules by IFN-γ on the PPCs was also examined by flow cytometry (Figure 3.4). Data revealed that 100% of untreated PPCs were detected positive against surface MHC-I expression and obvious shift of peak was observed in PPCs treated with IFN-γ, indicating that the average

expression of MHC-I molecules on cell surface was increased after incubation with IFN- γ. These experimental data demonstrated that PPCs were subjected to MHC-I induction by IFN- γ.

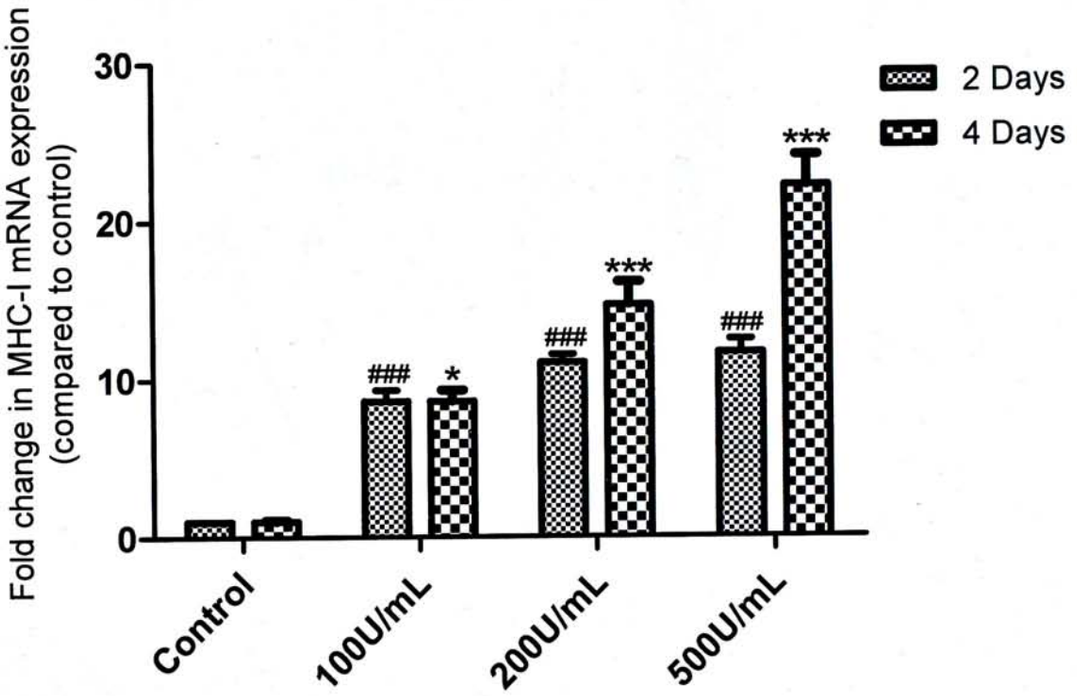

Figure 3.3 Real-time RT-PCR analyses of the mRNA expression of MHC-I molecules in IFN- γ treated PPCs. PPCs were cultured with 100, 200 and 500U/mL human recombinant IFN-γ for 2 and 4 days. The expression of MHC-I molecules in PPCs was significantly upregulated by IFN-γ at different concentrations (100U/mL, 200U/ml and 500U/mL) for different days (2 and 4 days). The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data were expressed as means ±SEM for three experiments in each group.*denotes $p<0.05$ when compared to 4-days control; ### and *** denote $p<0.001$ when compared to 2-days and 4-days control, respectively.

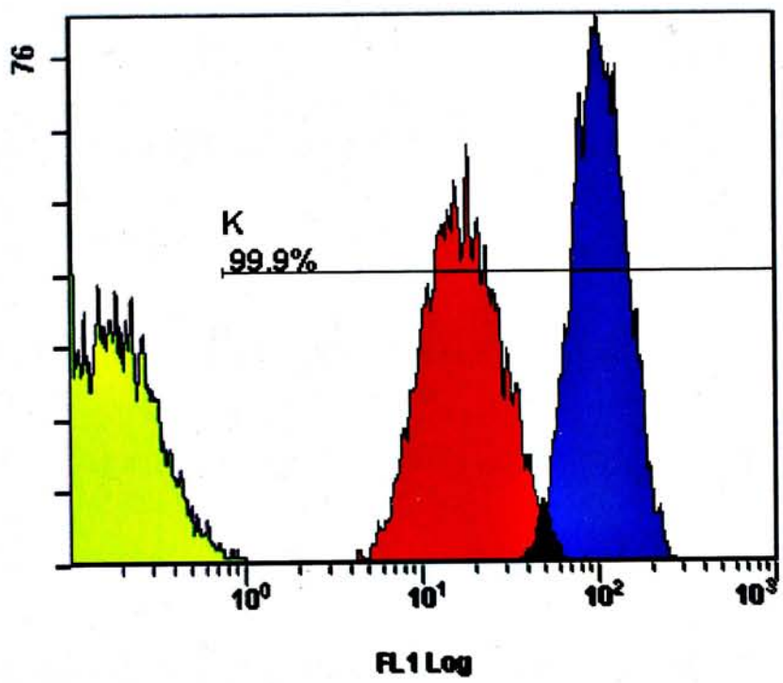

Figure 3.4 A representative histogram of the flow cytometric analysis of surface MHC-I expression in IFN- γ untreated and treated PPCs. PPCs were cultured with 200U/mL human recombinant IFN-γ for 2 days before flow cytometry analysis. The fluorescent intensity of MHC-I detection increase after pre-treatment of IFN-γ, suggesting an upregulation of MHC-I molecules on cell surface. The non-specific binding is normalized by the use of isotype antibody. Yellow: isotype control; Red: PPCs control; Blue: PPCs treated with 200U/mL IFN-γ for 48hr.

3.2.2 Effect of IFN-γ and IL-10 on HLA-G expression in PPCs and ICCs

The effect of IL-10 on HLA-G expression in PPCs and ICCs was evaluated by quantitative real-time PCR. PPCs and ICCs were incubated with 5, 25 and 100U/mL human recombinant IL-10 for 2 days. Results revealed that IL-10 upregulated HLA-G expression on PPCs (Figure 3.5) and ICCs (Figure 3.6), peaking at 25U/mL (5.99±1.39 and 3.18±0.07), respectively. Data implies the capacity of IL-10 on HLA-G induction in PPCs/ICCs. Western blot analyses were employed to investigate the effect of IFN- γ and IL-10 on the protein expression of HLA-G. A previous report demonstrated a predominant expression of HLA-G by the human placental choriocarcinoma cell line JEG-3 (Yie et al., 2006). Therefore, it was employed as positive control for HLA-G expression. JEG-3 cell line and PPCs were pretreated with 25U/mL IL-10 or 200U/mL IFN- γ for two days. Figure 3.7 revealed that HLA-G protein was upregulated in both PPCs and JEG-3 after IL-10 treatment, while the HLA-G protein remained unchanged in both PPCs and JEG-3 cells treated with IFN-γ.

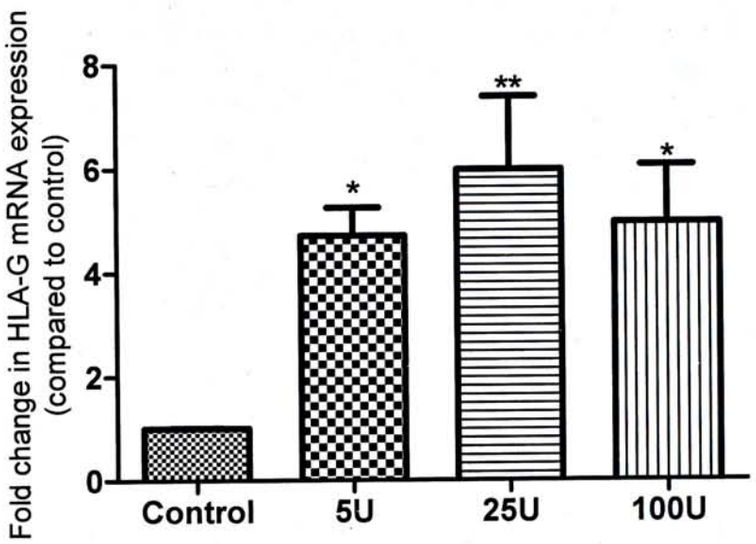

Figure 3.5 Real-time RT-PCR analyses of the mRNA expression of HLA-G on IL-10 treated PPCs. PPCs were cultured with 5, 25 and 100U/mL human recombinant IL-10 for 2 days. The expression of HLA-G gene was upregulated by IL-10, peaking at 25U/mL. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group.*denotes $p<0.05$ when compared to control; ** denotes $p<0.01$ when compared to control.

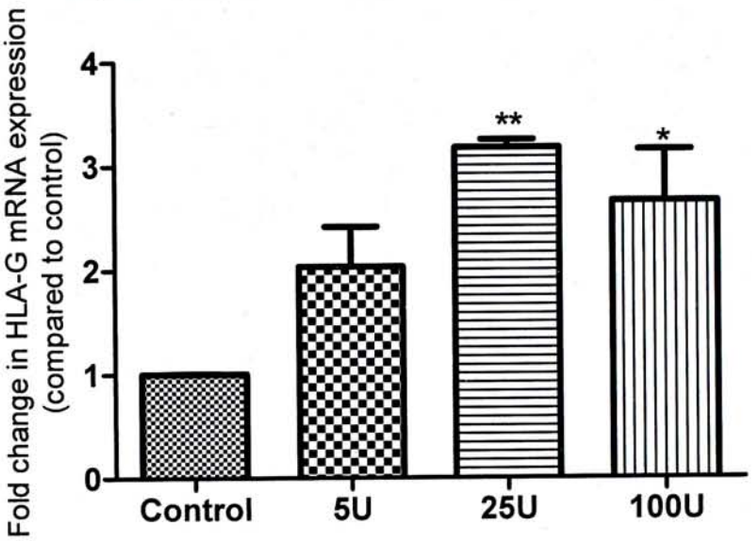

Figure 3.6 Real-time RT-PCR analysis of the mRNA expression of HLA-G on IL-10 treated ICCs. ICCs were cultured with 5, 25 and 100U/mL human recombinant IL-10 for 2 days. The expression of HLA-G gene on ICCs was upregulated by IL-10, peaking at 25U/mL. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group.*denotes $p<0.05$ when compared to control; ** denotes $p<0.01$ when compared to control.

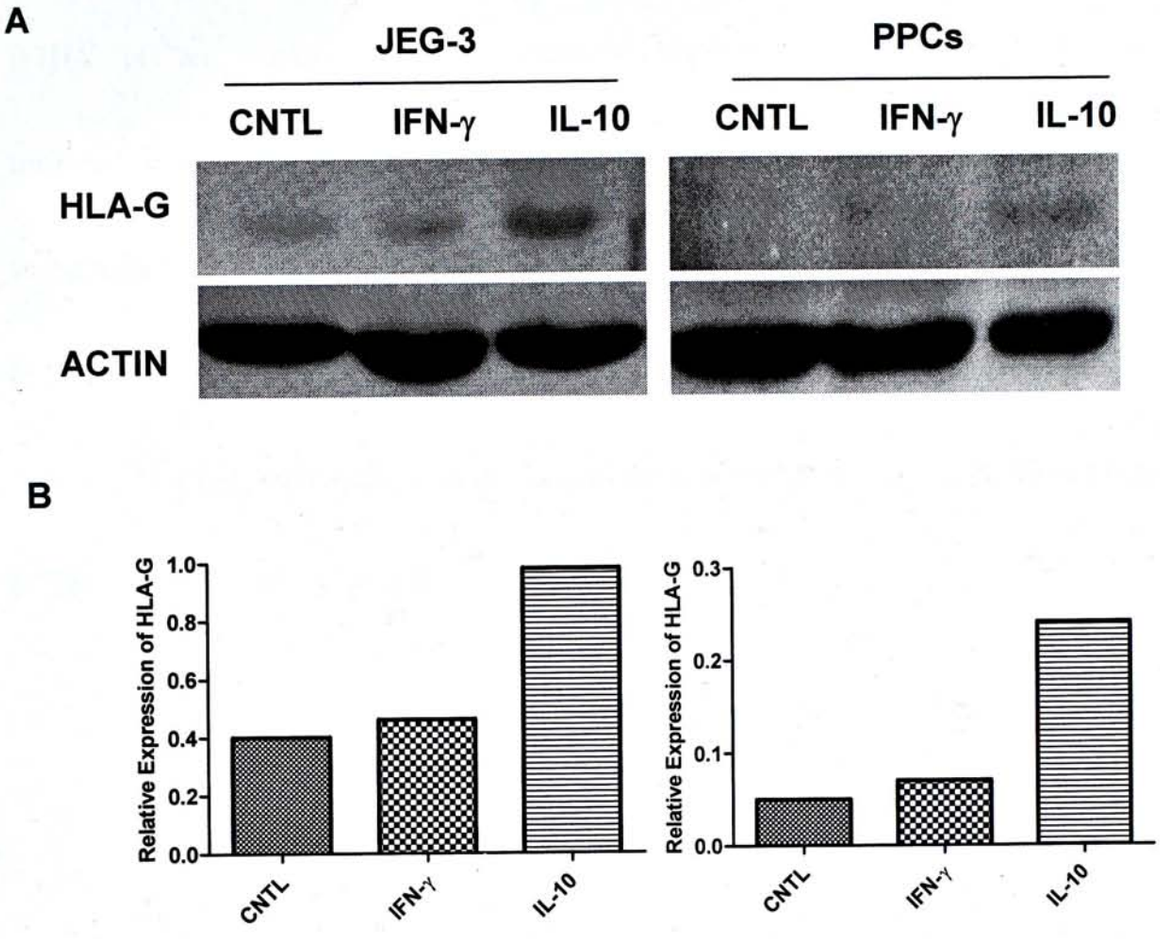

Figure 3.7 Western blotting of the HLA-G protein in JEG-3 and PPCs treated with 200U/mL IFN- γ and 25U/mL IL-10 for 2 days. JEG-3 was employed as the positive control. (A) HLA-G protein derived from different treatments. 20 microgram aliquots of protein were loaded in each well. (B) The relative expressions of HLA-G by JEG-3 and PPCs after treatment with IFN- γ or IL-10. HLA-G protein expression in PPCs is upregulated by IL-10, while unchanged by IFN- γ. The relative expression was normalized as percentage of β-actin.

3.2.2 Effect of IFN-γ on B7H4 expression in PPCs

B7H4 is an innate molecule recently shown to be taking part in the immuno-modulation process (Prasad et al., 2003). The expression has been reported to be affected by certain cytokines (Sica et al., 2003). PPCs were incubated with IFN- γ of concentrations 100, 200 and 500U/mL for 2 days for mRNA expression of B7H4. RT-PCR revealed a dose-dependent upregulation of immuno-suppressive B7H4 mRNA in PPCs by IFN- γ (Figure 3.8).

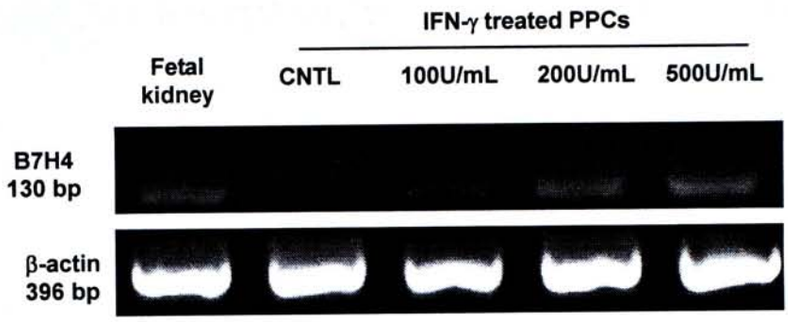

Figure 3.8 RT-PCR for mRNA expression of B7H4 on IFN- γ treated PPCs. PPCs were cultured with 100, 200 and 500U/mL human recombinant IFN-γ for 2 days. The expression of B7H4 on PPCs increased dose-dependently by IFN-γ. Fetal kidney was used as the positive control. RT-PCR for β-actin was used as housekeeping gene.

3.3 Comparison of immuno-properties of PPCs and ICCs from 1st and 2nd trimester

A recent study demonstrated a reduction of immunogenicity in human fetal pancreas (Brands et al., 2008). In this study a lowered MHC-I expression in PPCs than in fetal pancreas was noted. In this regard the immune-properties of PPCs derived from first (9-11 wk) and early second trimester (12-14wk) was analyzed.

3.3.1 Differential expression of MHC molecules in PPCs

The temporal expressions of MHC-I and II molecules in PPCs of different gestation weeks (9-14weeks) were evaluated by quantitative real-time PCR. Figure 3.8 revealed that no significant change in MHC-I expression across 9th to 12th week, however MHC-I remarkably increased in PPCs at 13th (4.18±0.584) and 14th week (5.30±1.21) when compared to that from 9th week.

Temporal expressions of MHC-II were also examined. MHC-II gene was upregulated at 10th week PPCs (22.77±1.82) compared to that from 9th week. The level continued to rise and peak at 12th week (103±5.29). Results showed a remarkable increase in the mRNA expression of MHC-II in PPCs derived from the second trimester.

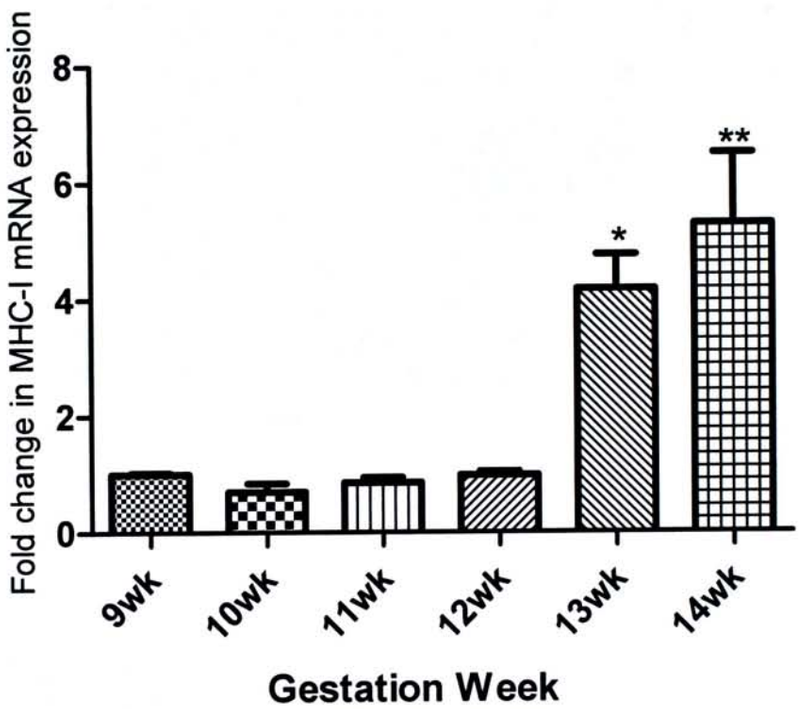

Figure 3.9 Real-time RT-PCR for mRNA expression of MHC-I in 9th-14th week PPCs. MHC-I gene was significantly upregulated at 13th week and 14th week. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group.*denotes $p<0.05$ when compared to 9 week PPCs; ** denotes $p<0.01$ when compared to 9 week.

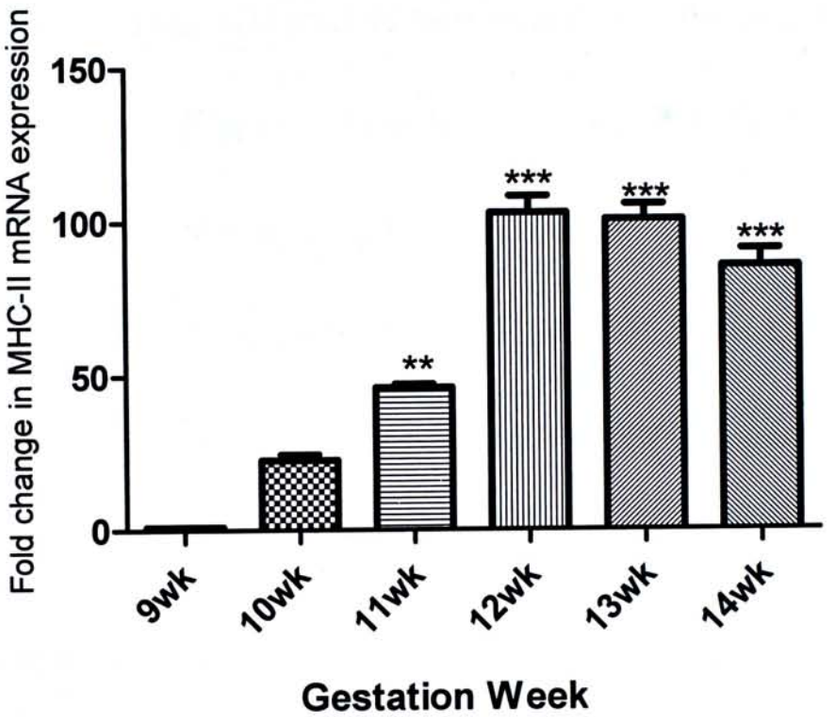

Figure 3.10 Real-time RT-PCR for mRNA expression of MHC-II in 9th-14th week PPCs. MHC-II gene was significantly upregulated 11th week onward, peaking at 12th week. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ** denotes $p<0.01$ when compared to 9 week PPCs; *** denotes $p<0.001$ when compared to 9 week.

3.3.2 Different immuno-related gene expression in PPCs and ICCs

To further elucidate the immunological difference between PPCs and ICCs at different stage of maturation, the mRNA expressions of several selected immune related genes were evaluated by real-time PCR. The mRNA expressions of pro-inflammatory (Figure 3.11) molecules namely, MHC-I, MHC-II, TNFSF10 and C3, were increased in PPCs at second trimester (MHC-I; 4.74±0.652, MHC-II; 91.3±7.87, TNFSF10; 104.8±19.5 and C3; 22.27±4.46). Among these 4 pro-inflammatory molecules, MHC-II and TNFSF10 showed a more drastic increase than MHC-I and C3. On the other hand, the mRNA expression of immune-suppressing molecules B7H4 and HLA-G were downregulated (B7H4; 0.02181±0.00519 and HLA-G; 0.486±0.0686) in PPCs of second trimester (Figure 3.12). Taken together, an upregulation of pro-inflammatory gene and down-regulation of immune-suppressive genes were noted in second trimester PPCs.

Similar results were obtained in different ICCs. A higher mRNA expression of MHC-I, MHC-II, C3, TNFSF 10 (1.58±0.0535, 3.546±0.253, 2.354±0.355 and 3.805±0.468 respectively) and lower level of B7H4 and HLA-G mRNA

(0.3187±0.0383 and 0.6512±0.1574 respectively) were detected in second trimester ICCs compared with those derived from first trimester(Figure 3.13 and 3.14). Interestingly, the fold differences of gene expressions were much less in ICCs than in PPCs.

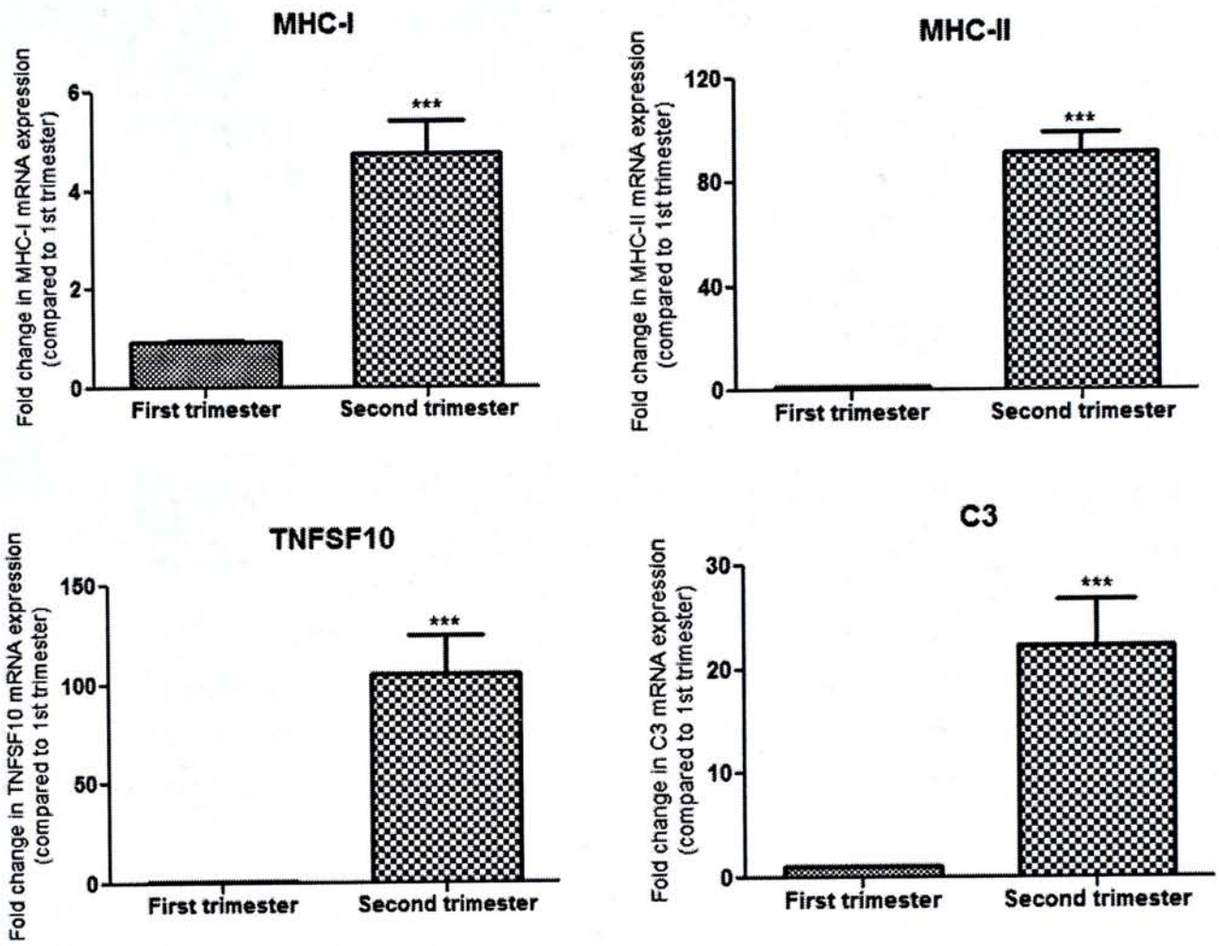

Figure 3.11 Real-time RT-PCR for mRNA expression of MHC-I, MHC-II, TNFSF10 and C3 in PPCs of first and early second trimester. The mRNA expression of pro-inflammatory molecules namely MHC-I, MHC-II, TNFSF10 and C3 were found significantly increased in PPCs of second trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. *** denotes $p<0.001$ when compared to first trimester.

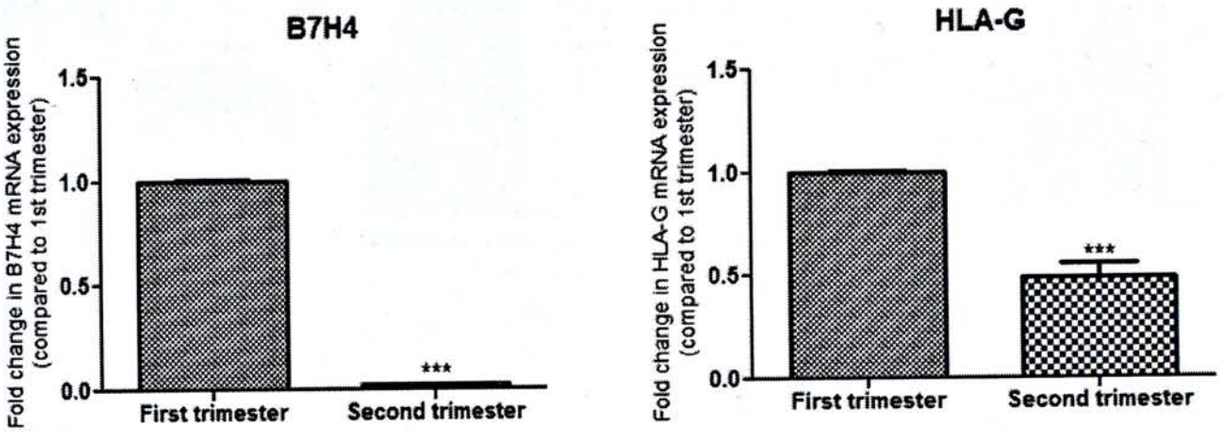

Figure 3.12 Real-time RT-PCR for mRNA expression of B7H4 and HLA-G in PPCs of first and early second trimester. The mRNA expressions of immune-suppressive molecules namely, B7H4 and HLA-G, were found significantly decreased in PPCs of second trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. *** denotes $p<0.001$ when compared to first trimester.

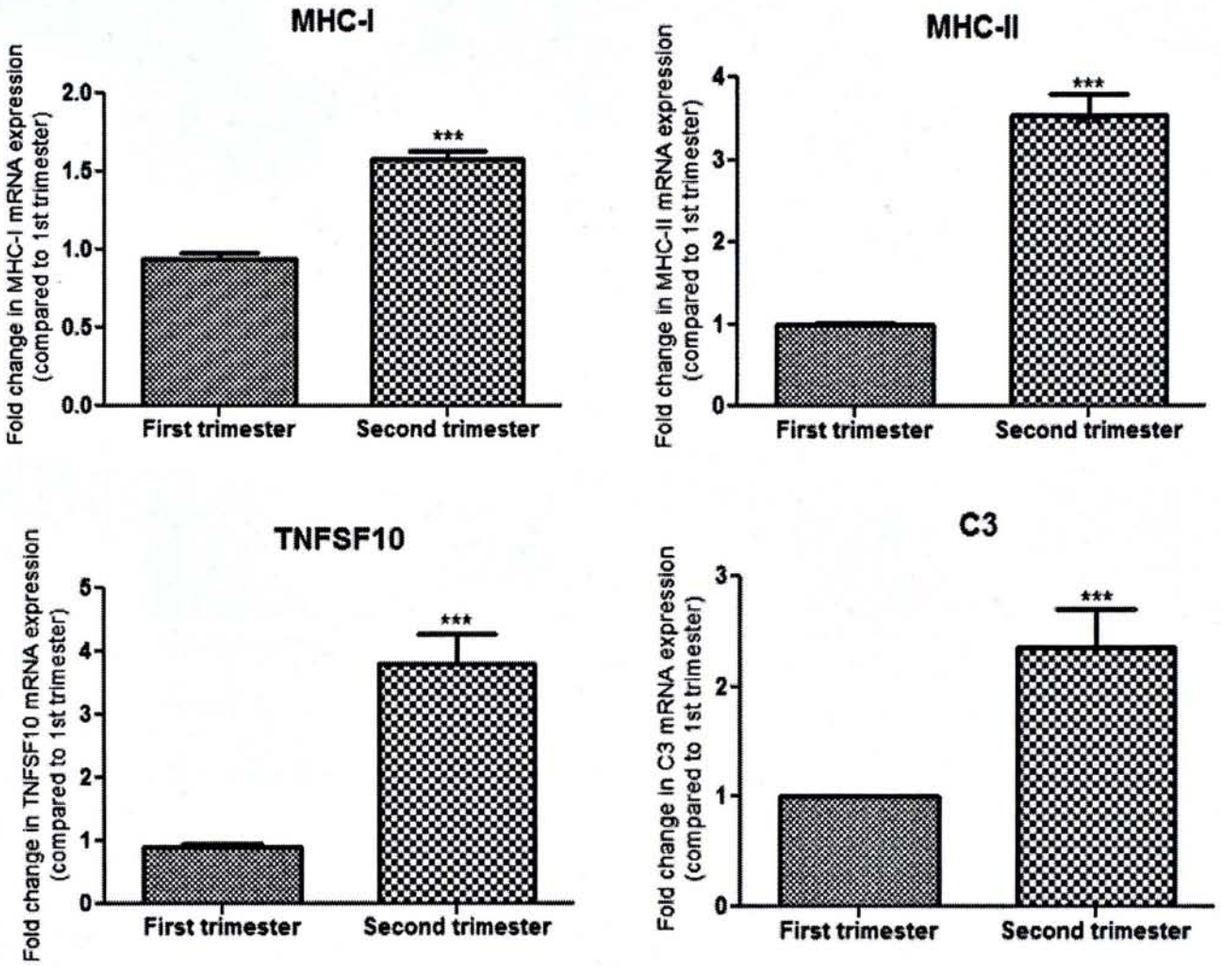

Figure 3.13 Real-time RT-PCR for mRNA expression of MHC-I, MHC-II, TNFSF10 and C3 in ICCs of first and early second trimester. The mRNA expression of pro-inflammatory molecules namely, MHC-I, MHC-II, TNFSF10 and C3 were found significantly increased in ICCs of second trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. *** denotes $p<0.001$ when compared to first trimester.

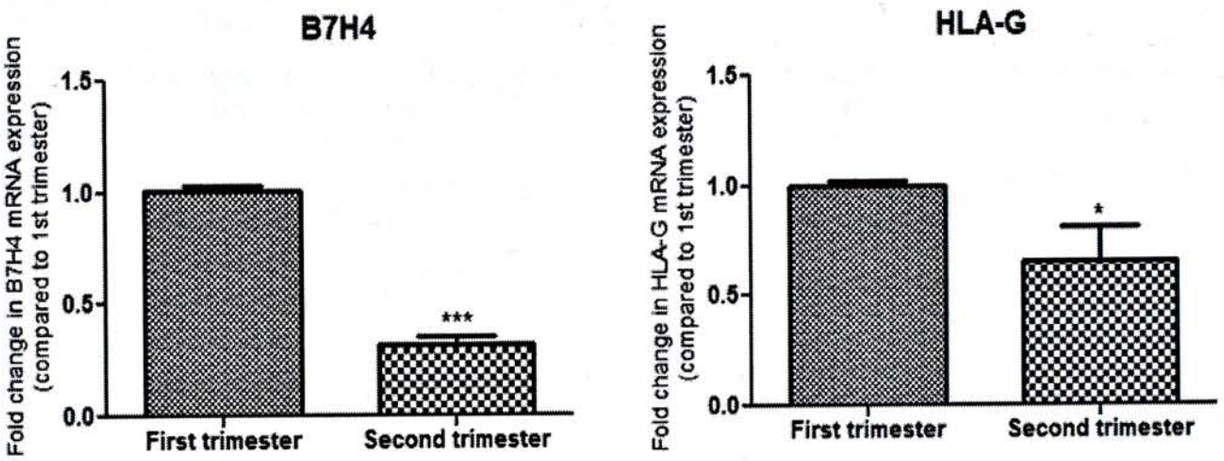

Figure 3.14 Real-time RT-PCR for mRNA expression of B7H4 and HLA-G in ICCs of first and early second trimester. The mRNA expression of immune-suppressive molecules namely, B7H4 and HLA-G, were found significantly decreased in ICCs of second trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. * denotes $p<0.05$ when compared to first trimester; *** denotes $p<0.001$ when compared to first trimester.

3.3.3 Comparison of IFN-γ-activated MHC expression in PPCs and ICCs

As shown in the study, the cytokine IFN-γ was capable of inducing both the transcription and translation of MHC on PPCs and ICCs. It was unclear of any difference between the first and second trimester. Figure 3.15 and Figure 3.17 show that IFN- γ induced a significant higher MHC-I mRNA expression in second trimester PPCs and ICCs than in first trimester PPCs and ICCs (16.77±0.383 vs 21.12±0.482 in PPCs and 2.05±0.0924 vs 4.34±0.241 in ICCs). Besides, lower extent of MHC-I upregulation was noted in ICCs than PPCs. Flow cytometry analyses display a greater degree of right shift of positive signals in second trimester PPCs and ICCs than the first one in both PPCs (Figure 3.16) and ICCs (Figure 3.18), suggesting that IFN-γ induced MHC-I transcription and translation in second-trimester PPCs and ICCs.

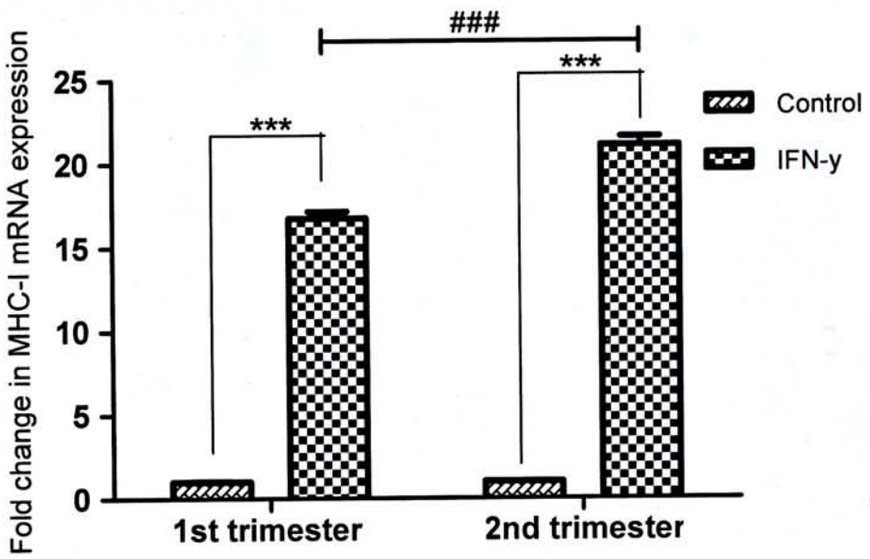

Figure 3.15 Real-time RT-PCR for mRNA expression of MHC-I in IFN-γ induced PPCs of first and second trimester. Both groups showed an increased MHC-I expression after treatment of 200U/mL IFN-γ for 2 days. The degree of upregualation in the gene expression of MHC-I was significantly higher in second trimester PPCs. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ***denotes $p<0.001$ when compared to control; ### denotes $p<0.001$ when compared to IFN-γ treatment in first trimester.

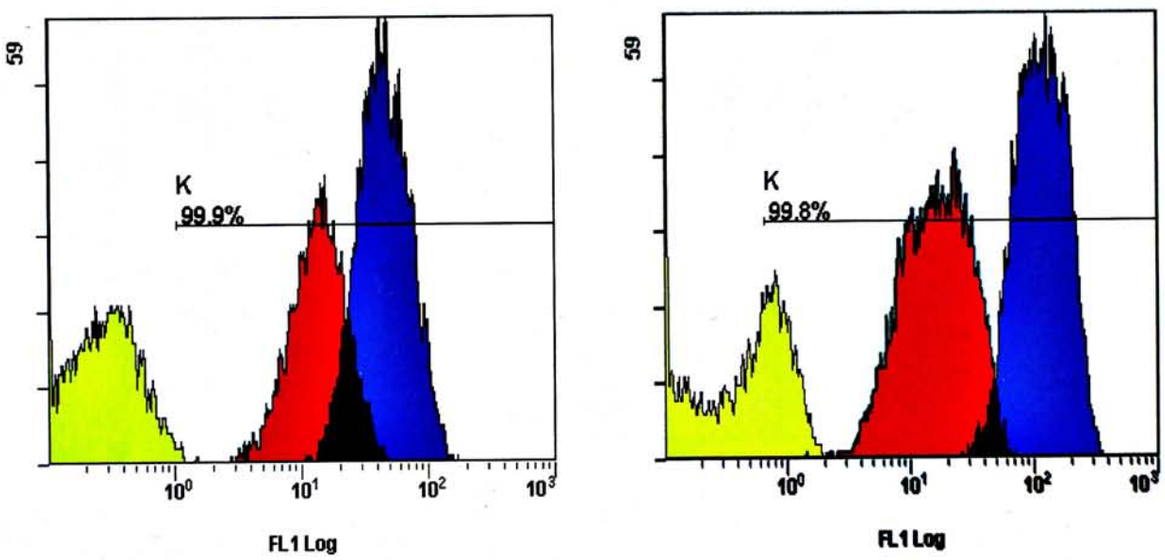

Figure 3.16 Flow cytometry analysis of surface MHC-I expression on IFN-γ treated PPCs. Both PPCs of (A) first and (B) second trimester were treated with 200U/mL IFN-γ for 2 days. Yellow: isotype control; Red: PPC control; Blue: IFN-γ treated PPC. Results revealed that a right shift of the signal intensity of MHC-I in treated PPCs. The shift of fluorescent intensity is more remarkable in second trimester PPCs. The non-specific binding is normalized by the used of isotype antibody.

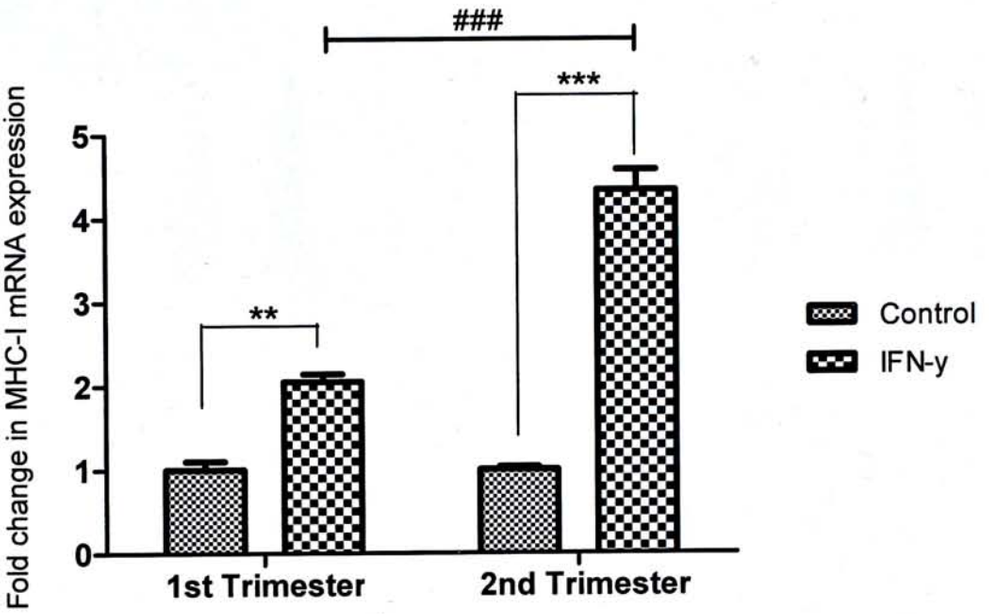

Figure 3.17 Real-time RT-PCR for mRNA expression of MHC-I in IFN-γ ICCs of first and second trimester. Both groups showed an increased MHC-I molecules expression after treatment of 200U/mL IFN-γ for 2 days. The degree of upregualation in the gene expression of MHC-I was significantly higher in second trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ***denotes $p<0.001$ when compared to control; ### denotes $p<0.001$ when compared to IFN-γ treatment in first trimester.

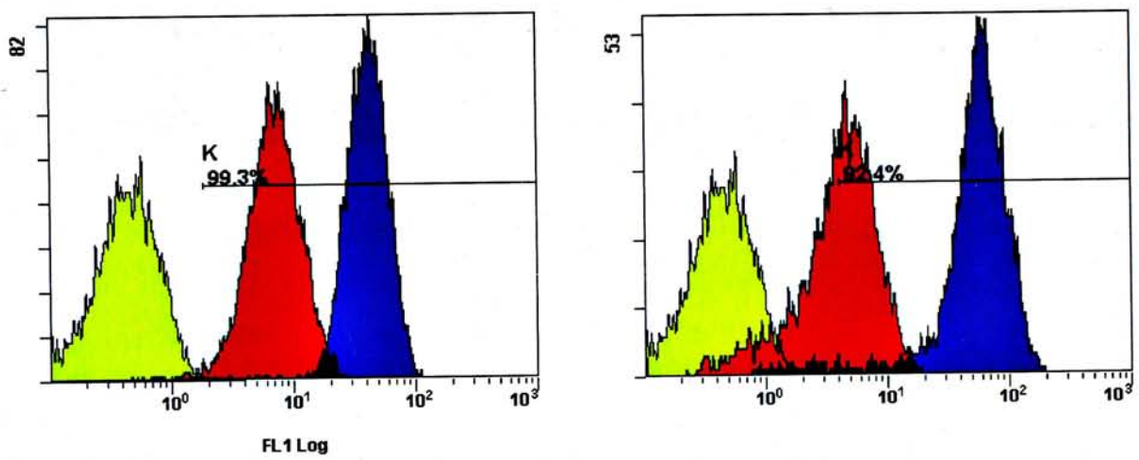

Figure 3.18 Flow cytometry analysis of surface MHC-I expression on IFN-γ treated ICCs. Both ICCs of (A) first and (B) second trimester were treated with 200U/mL IFN-γ for 2 days. Yellow: isotype control; Red: PPC control; Blue: IFN-γ treated PPC. Results revealed that a right shift of the sgnal intensity of MHC-I molecules in treated ICCs. The shift of fluorescent intensity is more remarkable in second trimester ICCs. The non-specific binding is normalized by the used of isotype antibody.

3.3.4 Comparison of other IFN-γ activated genes expression in PPCs

Apart from MHC-I, the effect of IFN- γ on gene expression of MHC-II, HLA-G and B7H4 in PPCs were also investigated. Surprisingly, after treatment with 200U/mL IFN- γ for 2 days, the expression of MHC-II was drastically increased in PPCs (Figure 3.19), with over 50000 folds for first trimester (55340±17700) and over 150000 folds for second trimester (188900±27000). The data showed that IFN- γ was a potent inducer of MHC-II in PPCs.

In contrast, the upregulation of mRNA expression of HLA-G and B7H4 by IFN- γ were not as drastic as MHC-II in PPCs. A two-fold increase in HLA-G mRNA expression was detected in PPCs of first trimester upon stimulation of IFN- γ, while no observable change was detected in second trimester (Figure 3.20). IFN- γ also mildly induced B7H4 mRNA expression in the first and second trimester PPCs, <2 folds in 1st trimester. The up-regulation of HLA-G and B7H4 in PPCs by IFN- γ suggested the potential immune-suppressive nature of PPCs upon cytokine challenges, which may help negatively regulate the immune response.

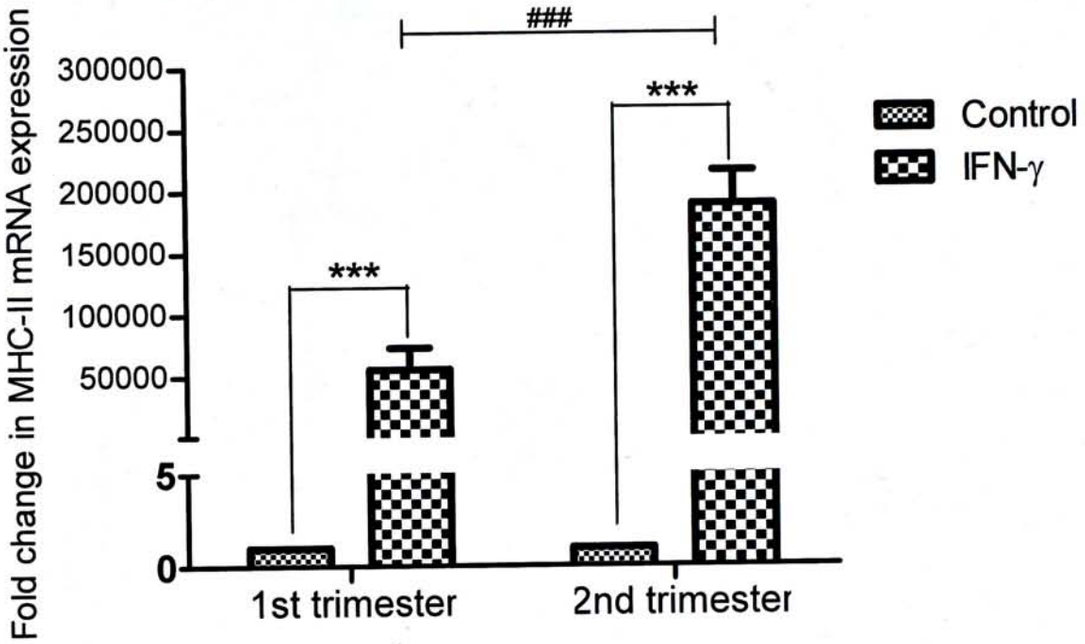

Figure 3.19 Real-time RT-PCR for mRNA expression of MHC-II in IFN-γ induced PPCs of first and second trimester. Both groups showed an increased MHC-I expression after treatment of 200U/mL IFN-γ for 2 days. The degree of upregulation in the gene expression of MHC-II was significantly higher in second trimester (>150,000 fold). The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ***denotes $p<0.001$ when compared to control; ### denotes $p<0.001$ when compared to IFN-γ treatment in first trimester.

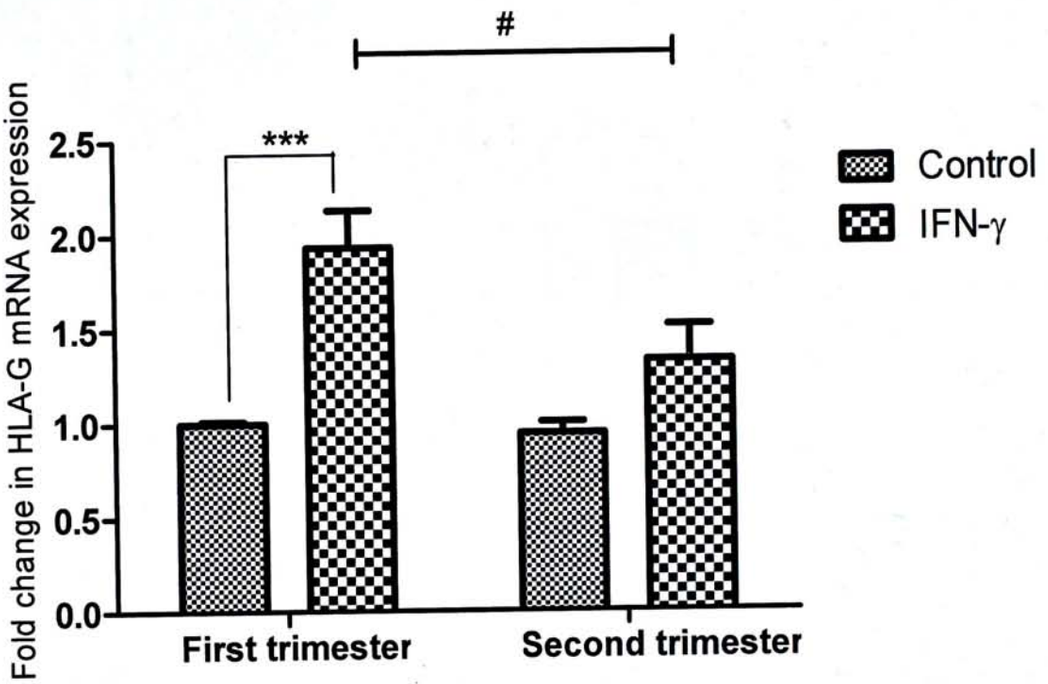

Figure 3.20 Real-time RT-PCR for mRNA expression of HLA-G in IFN-γ induced PPCs of first and second trimester. Both groups showed an increased MHC-I molecules expression after treatment of 200U/mL IFN-γ for 2 days. The degree of upregulation in the expression of MHC-I molecules was significantly higher in first trimester. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. ***denotes $p<0.001$ when compared to control; # denotes $p<0.05$ when compared to IFN-γ treatment in first trimester.

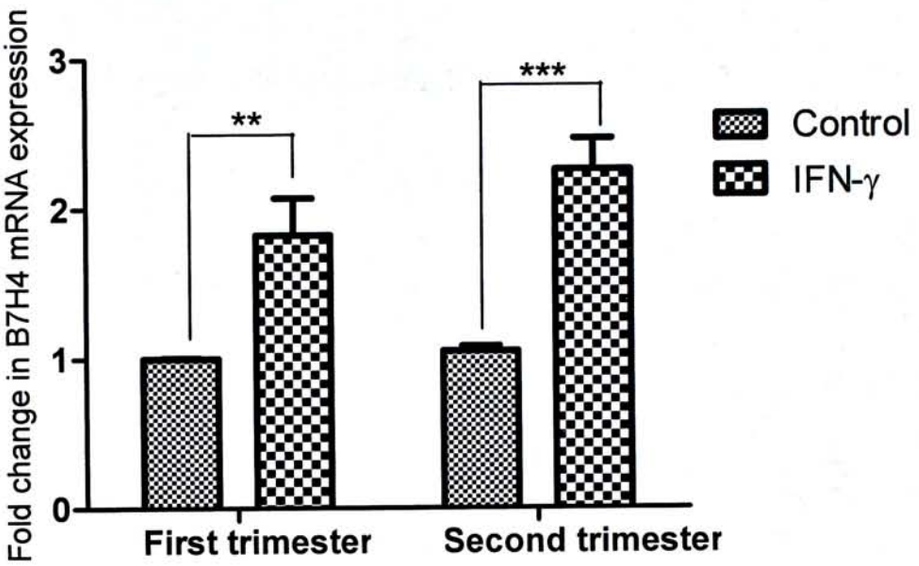

Figure 3.21 Real-time RT-PCR for mRNA expression of B7H4 in IFN-γ induced PPCs of first and second trimester. Both groups showed an increased B7H4 expression after treatment of 200U/mL IFN-γ for 2 days. The relative expression was normalized as a percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta CT}$. All data are expressed as means ±SEM for three experiments in each group. **denotes $p<0.01$ when compared to control; *** denotes $p<0.001$ when compared to control.

3.4Mixed lymphocyte reaction of PPCs from 1st and 2nd trimester

3.4.1 Effect of PPCs on proliferation of PBMC

The mixed lymphocyte reaction (MLR) is the in vitro assessment of the immunogenicity of cells by co-culturing the target cells with the effector cells, and the proliferation of the target cells is assessed. The set-up has recently been used to evaluate the immunological status of different types of stem cells, such as MSCs, ESCs and neural stem cells as elsewhere reported. PPCs (5×10^2, 5×10^3, 5×10^4 and 1×10^5 cells/well) were co-cultured with 2×10^5 isolated human PBMCs. BrdU incorporation assay revealed a significant increase in PBMC proliferation upon co-culture with PPCs at 5×10^3 cells/well or above (Figure 3.22). Proliferation of PBMCs exhibited a peak at 5×10^4 cells/well PPCs (150±9.89%), and slightly dropped when the cell number reached 1×10^5 (128.1±6.23%). It would be attributed to the suboptimal culturing, which adversely effected the viability of PBMCs. In general, data showed that the cell number of PPCs present in the co-culture system correlated to the extent of proliferation of PBMCs, suggesting that PPCs could trigger the proliferation of PBMCs.

The MLR were then performed using PPCs of first and second trimester (Figure 3.23). PPCs of different trimesters, either with or without cytokine pretreatment,

were co-cultured with PBMCs for 6 days. Irradiated PPCs showed minimal BrdU incorporation and pre-treatment of either IFN-γ or IL-10 did not pose any significant effect on proliferation of PPCs. The co-culture of PBMCs with PPCs of both first and second trimester induced a significant PBMC proliferation, compared to PBMC alone. A higher extent of BrdU incorporation was observed in co-cultures of PBMCs and PPCs of second trimester (120.6±7.83% vs 140.6±3.79%). Early data of this study showed that IFN-γ could upregulate the MHC expression in PPCs. MLR results attested that IFN-γ potentiated PPCs at the first and second trimesters inducing proliferation of PBMCs, via the upregulation of MHC on PPCs. Similarly, the IFN-γ-treated PPCs of second trimester elicited a more vigorous proliferation in PBMCs than first trimester PPCs (143.9±7.85% vs 241.5±7.57%).

PPCs of both trimesters were primed with IL-10 prior to MLR. Results revealed that the stimulatory effects of first trimester PPCs on PBMC proliferation were abolished with IL-10 (98.52±4.06%). However, the amelioration was not so obvious in second trimester PPCs (125.8±9.42%). To note, all the PPCs groups, irrespective of IFN-γ and IL-10 treatment, exerted a lower proliferation on PBMCs, compared to the allogenic control (353.7±19.8%).

3.4.2 Effect of ICCs on proliferation of PBMC

Figure 3.24 displays the BrdU incorporation of PBMCs in co-culture of PBMCs with ICCs of second trimester. An enhanced BrdU incorporation was noted in co-cultures with and without IFN-γ priming of ICCs (140.0±10.94%)

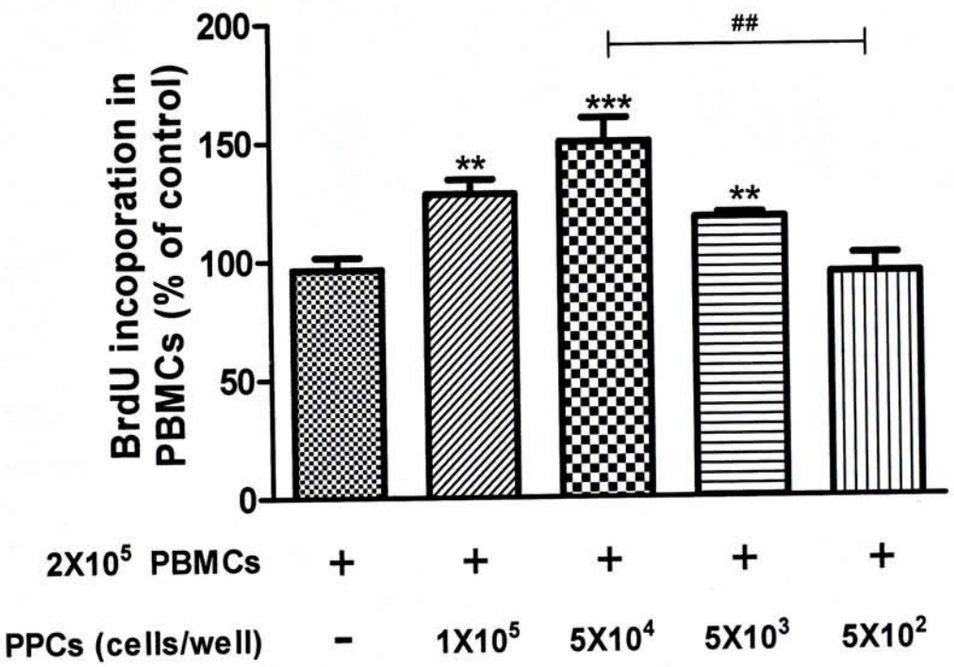

Figure 3.22 Brdu incorporation analyses of human PBMCs post mixed lymphocyte reaction. BrdU incorporation assay revealed a significant increase of BrdU incorporation in PBMCs upon the co-culture with PPCs at $5X10^3$ cells/well or above. The proliferation of PBMCs increased with the increase of PPCs ($5X10^2$- $5X10^4$), and peaked at $5X10^4$ cells/well. The relative expression was normalized as a percentage of control. All data are expressed as means $\pm$SEM for six experiments in each group. **denotes $p<0.005$ when compared to PBMC alone; ***denotes $p<0.001$ when compared to PBMC alone; ## denotes $p<0.005$ when compared to the lowest cell number.

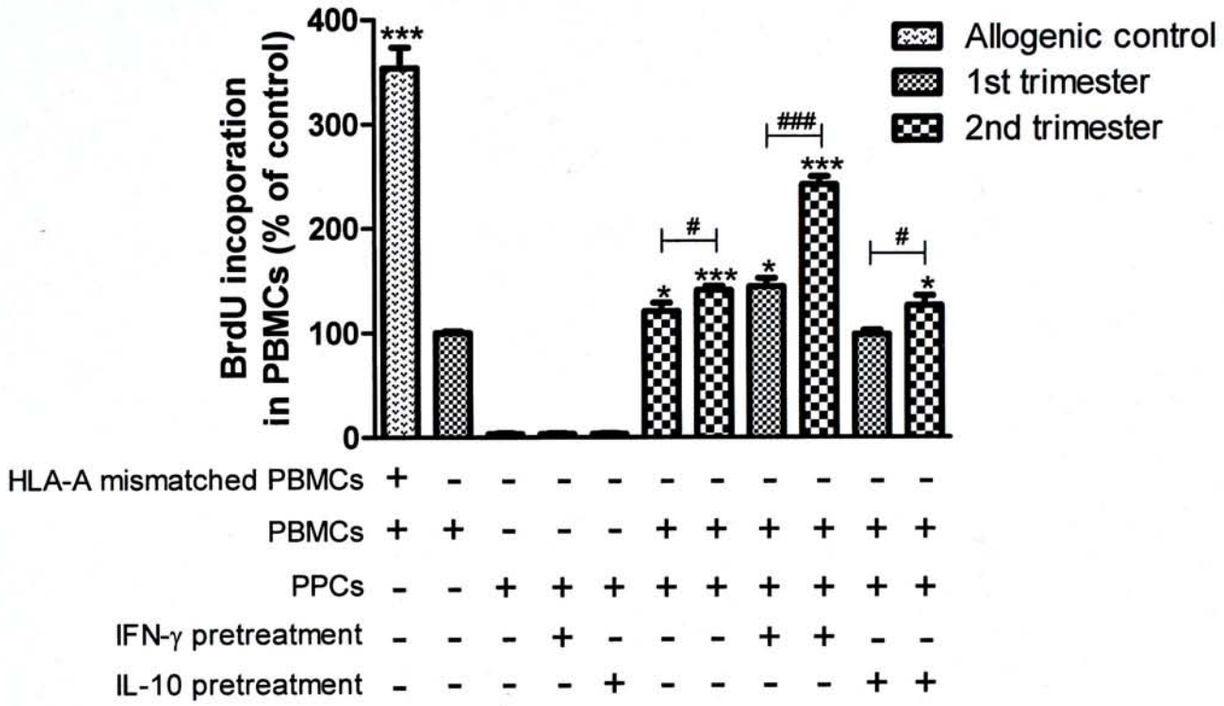

Figure 3.23 Brdu incorporation analyses of human PBMCs post mixed lymphocyte reaction. BrdU incorporation assay revealed an increased proliferation of PBMC when co-culturing with PPCs of second trimester. The extent of proliferation of PBMCs were much higher when PPCs were pretreated with 200U/mL IFN- γ for 2 days. The addition of IL-10 to PPCs tended to abolish the stimulative effect of first trimester PPCs of first trimester, however no evidence on second trimester PPCs. The degree of proliferation of PBMCs elicited by PPCs of all groups were lower than allogenic control. The relative expression was normalized as a percentage of the PBMCs control. All data are expressed as means ±SEM for six experiments in each group. *denotes $p<0.05$ when compared to PBMC alone; ***denotes $p<0.001$ when compared to PBMC alone; # denotes $p<0.05$ when compared to according PPC of first trimester; ## denotes $p<0.005$ when compared to according PPC of first trimester.

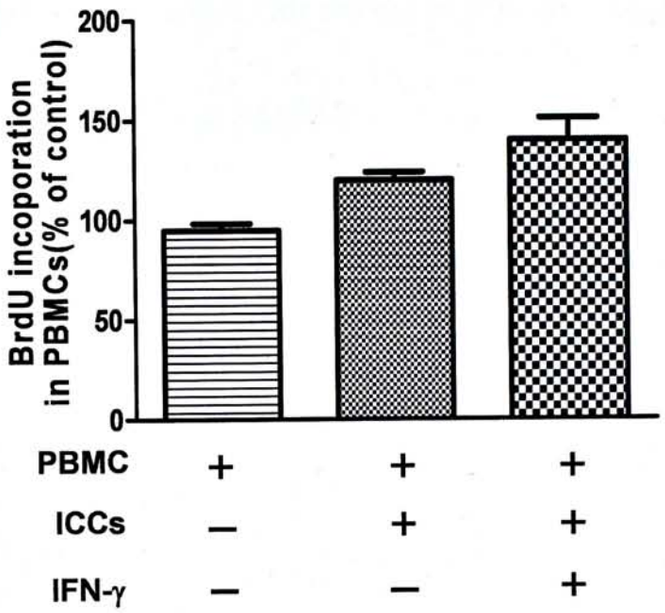

Figure 3.24 BrdU incorporation analyses of human PBMCs post mixed lymphocyte reaction. BrdU incorporation assay revealed an increased proliferation of PBMC when co-culturing with ICCs of second trimester (n=2). The degree of proliferation of PBMCs were more remarkable when ICCs were pretreated with 200U/mL IFN- γ for 2 days. The relative expression was normalized as percentage of the according control.

3.4.3 Effect of PPCs on Cytokines production in PBMC

MLR were also performed in the presence of lymphocyte stimulating factor, PHA-P, to investigate the cytokine release profile upon co-culture with PPCs at the first and second trimesters. ELISA showed a basal release of IFN- γ (3024±76.0 pg/mL) from PBMCs when stimulated with PHA-P (Figure 3.25). There was a significant surge of IFN- γ in co-cultures of PHA-P stimulated PBMCs and PPCs at the first and second trimester (3493±251 pg/mL for first trimester vs 5642±130 pg/mL for second trimester).

The level of IL-10 was also evaluated (Figure 3.26). ELISA revealed the production of IL-10 from PBMCs after stimulation of PHA-P (4019±315 pg/ml). PPCs at first- and second- trimester prohibited the production of IL-10 from PHA-P stimulated PBMCs into the MLR cultures (First trimester PPCs: 2368±144.3, second trimester PPCs: 1748±129.7 pg/ml).

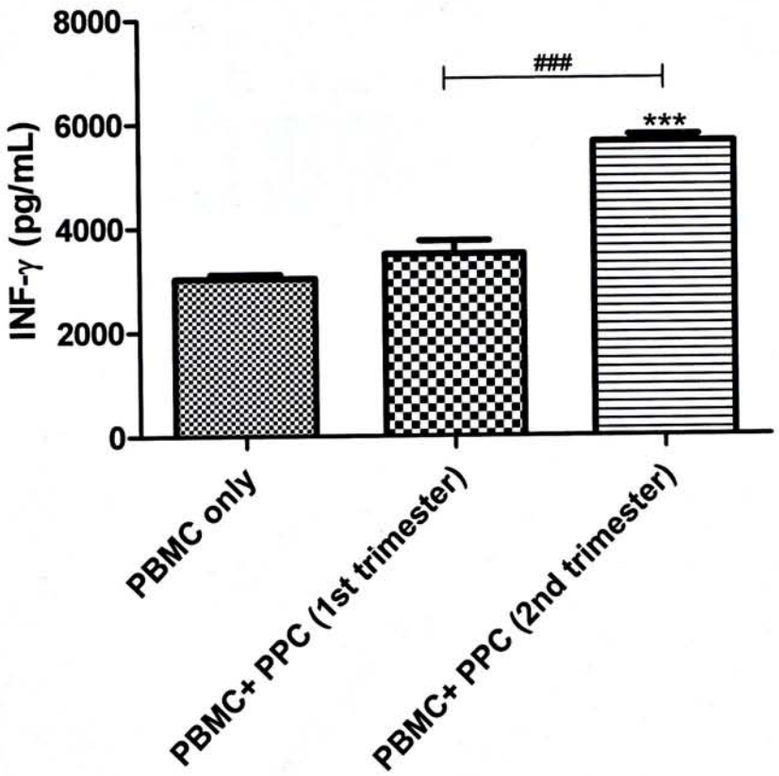

Figure 3.25 ELISA of IFN-γ in spent media collected from MLR of PHA-P-stimulated PBMCs and PPCs. IFN-γ (~3000 pg/mL) was detected in the spent media of PBMC cultures stimulated by PHA-P. IFN-γ was significantly increased in co-cultures of PPCs of second trimester and PHA-P stimulated PBMCs. All data are expressed as means ±SEM for four experiments in each group. ***denotes $p<0.001$ when compared to PBMC alone; ## denotes $p<0.001$ when compared to PPCs of first trimester.

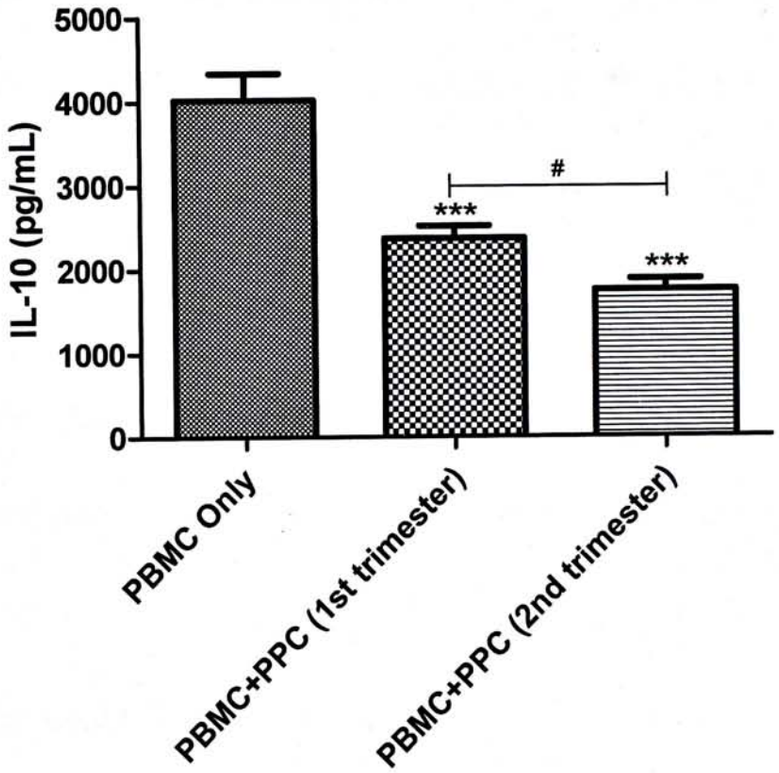

Figure 3.26 ELISA of IL-10 in spent media collected from MLR of PHA-P stimulated-PBMCs and PPCs. IL-10 (~4000 pg/mL) was detected in media of PBMC cultures stimulated by PHA-P. IL-10 was significantly decreased in co-cultures of PPCs of both groups and PHA-P-stimulated PBMCs. The inhibitory effect of PPCs at second trimester was more significant than first trimester. All data are expressed as means ±SEM for four experiments in each group. ***denotes $p<0.001$ when compared to PBMC alone; # denotes $p<0.05$ when compared to PPCs of first trimester.

3.5 Xenotransplantation of ICCs into diabetic mouse model

3.5.1 Blood glucose levels of diabetic mice after transplantation

To investigate the immune response evoked by ICCs in a xenogeneic environment, ICCs derived from PPCs of first and second trimester were transplanted into the left kidney capsule of 22 STZ-induced diabetic C57/BL6 mice. To determine the capacity of ICCs of different trimesters to control hyperglycemia, the blood glucose levels of diabetic mice were monitored before and 2 weeks after transplantation (Figure 3.27A). Data revealed a drastic drop of blood glucose, in both transplanted groups two days after transplantation (First trimester: 30.74±1.103 mmol/L on day 1 vs 23.84±1.78 mmol/L on day 2; Second trimester: 29.24±2.43 mmol/L on day 1 vs 26.82±2.32 mmol/L on day 2), compared to sham operated diabetic mice. Blood glucose started to rebound on day 3 in mice transplanted with second trimester ICCs and persisted at a high level for 2 weeks. The blood glucose of mice undergone transplant of PPCs at the first trimester remained at a comparable level on day 5. Both first and second trimester ICCs failed to normalize blood glucose in this xenotransplantation setting. However, the blood glucose levels of mice transplanted with first trimester grafts were significantly lower than those having second trimester graft on day 3 (Figure

3.27B). Blood glucose levels were slightly lower than that transplanted with second trimester grafts since then.

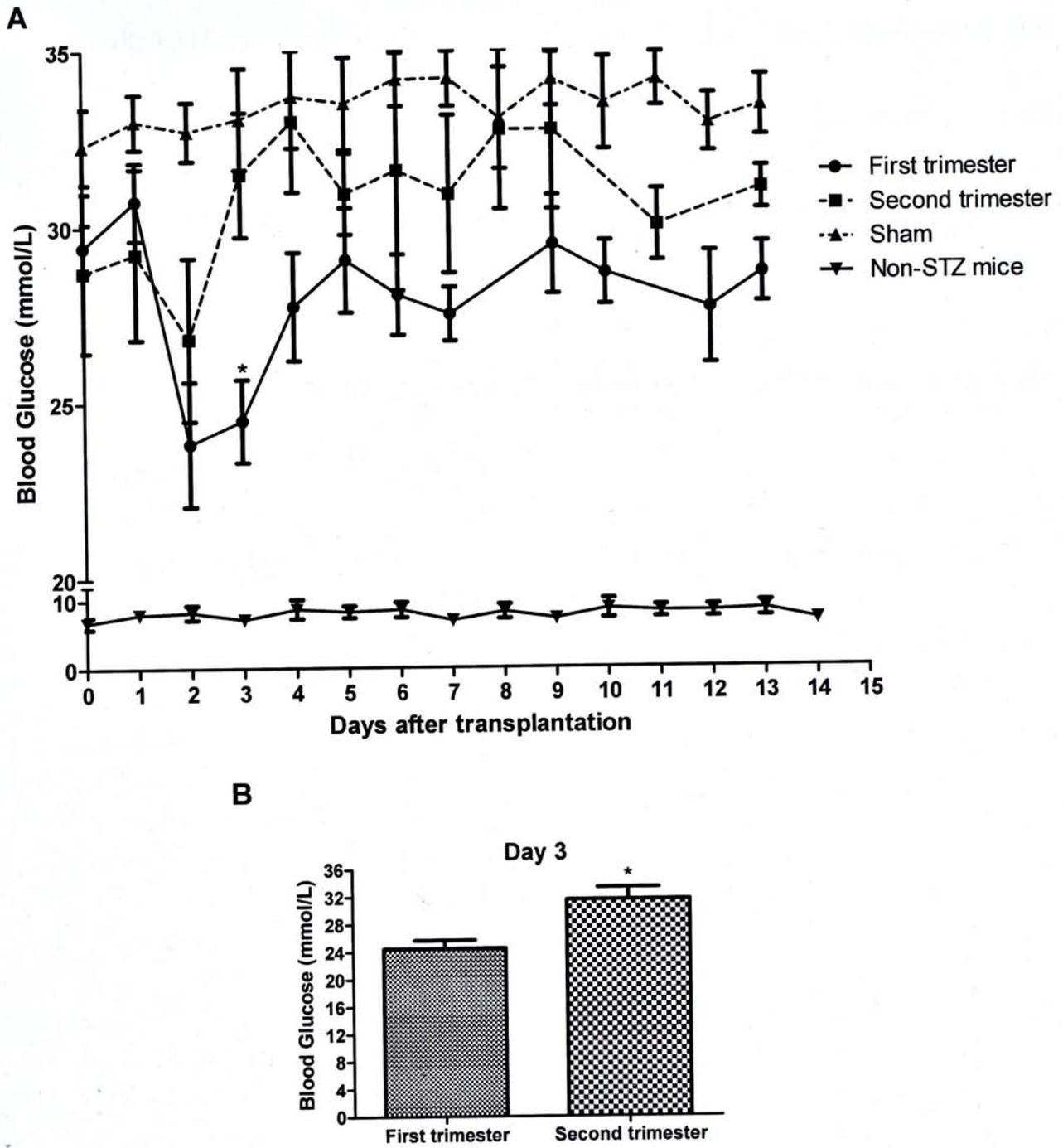

Figure 3.27 (A) Blood glucose level of 22 STZ-induced diabetic C57/BL6 mice after transplanted with ICCs of first and second trimester. Blood glucose levels of transplanted mice were monitored for two weeks. Mice transplanted with sterilized PBS were used as sham control. * denotes $p<0.05$ when compared to second trimester. (B) Blood glucose level of mice transplanted with first- and second- trimester grafts 3 days after transplantation. * denotes $p<0.05$ when compared to second trimester.

3.5.2 Histological evaluation of transplanted ICCs grafts

Grafted kidneys were harvested on 2, 4, 7 and 12 days after transplantation for histological examination. H-E staining revealed the intact graft under the renal capsule on day 2 and 4 post-transplant (Figure 3.28). Successful engraftment was evident one week post transplant (Figure 3.29A and B). However, on day 12, allo grafts diminished in size significantly (Figure 3.29C and D), suggesting graft rejection.

Immunohistochemical staining demonstrated the mature β-cell marker, PDX-1, in the renal capsule of mice undergone ICC transplant for one week, suggesting the successful engraftment of ICCs.

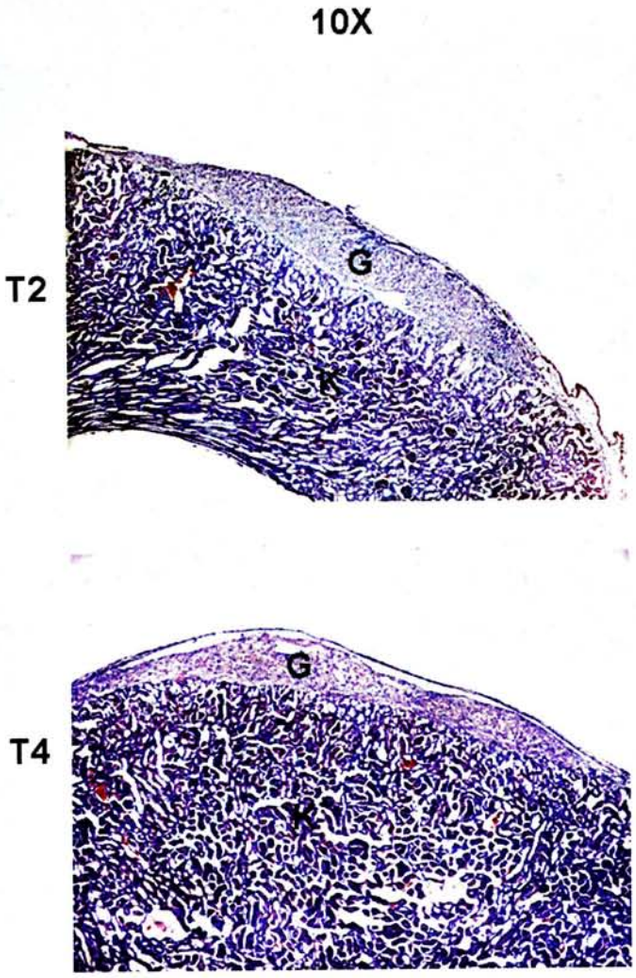

Figure 3.28 Histology of grafted kidneys harvested from adult STZ-induced C57/BL6 diabetic mice having undergone transplant. Representative images of a 13-week-old graft are shown. A: H-E staining of the grafts (2 days post transplant) at 10X magnification. B: H-E staining of the graft (4 days post transplant) at 10X magnification. G, human fetal ICCs graft; K, murine kidney.

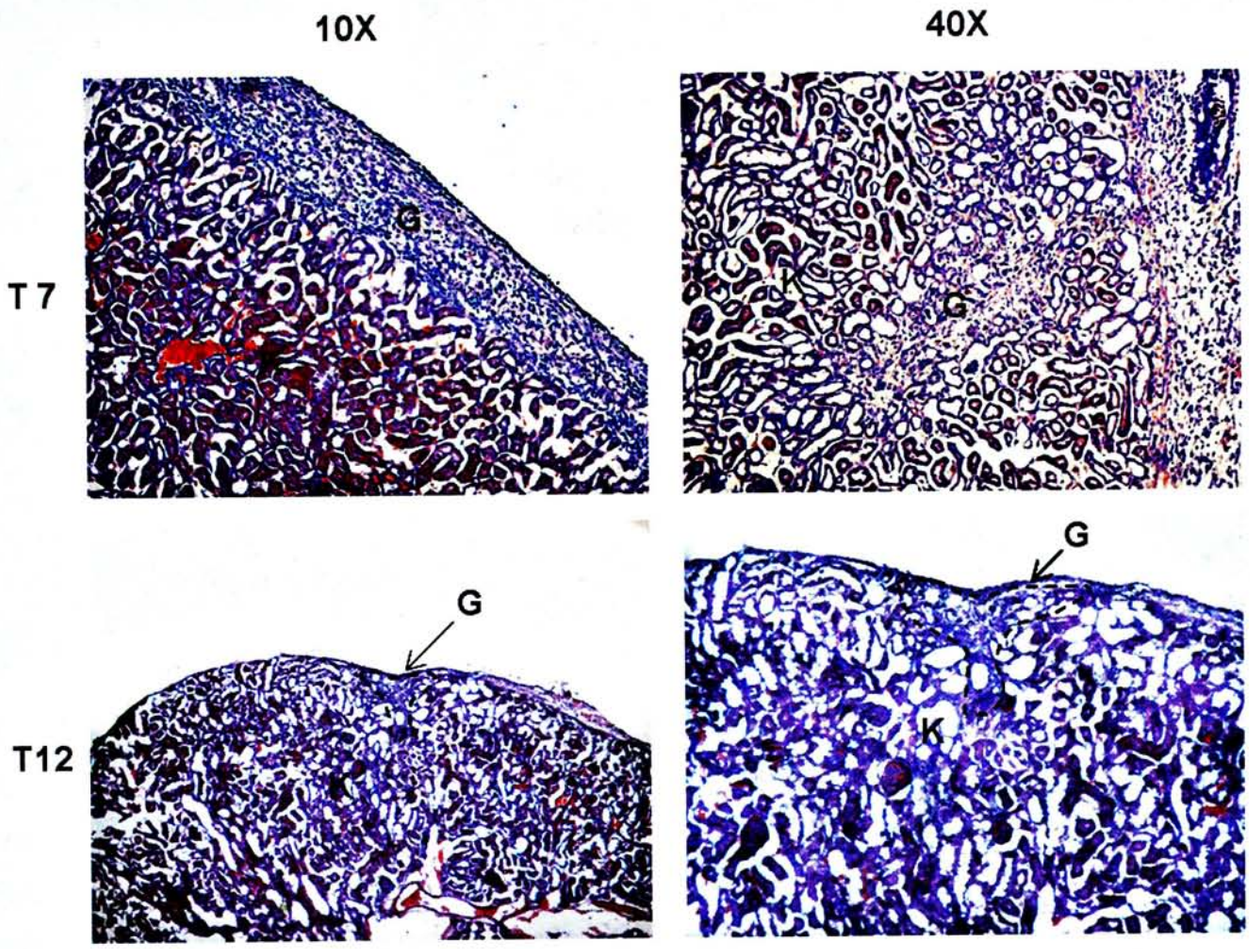

Figure 3.29 Histology of grafted kidneys were harvested from adult STZ-induced C57/BL6 diabetic mice having undergone ICC transplant. Representative image of a 13-week-old graft are shown. A: H-E staining of the grafts (7 days post transplant) at 10X magnification. B: H-E staining of the graft (7 days post transplant) at 40X magnification. Human ICC graft was evidnet. C: H-E staining of the graft (12 days post transplant) at 10X magnification. D: H-E staining of the graft (12 days post transplant) at 40X magnification. The size of ICCs graft diminished significantly. G, human fetal ICCs graft; K, murine kidney.

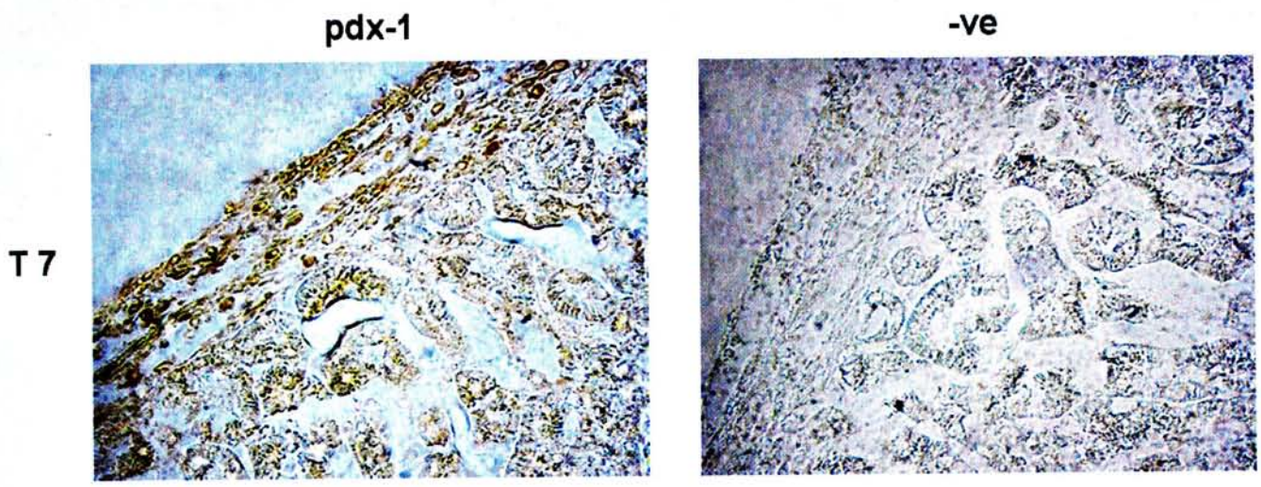

Figure 3.30 Immunohistochemical staining of human PDX-1. Grafted kidneys were harvested from adult STZ-induced C57/BL6 diabetic mice having undergone ICC transplant on day 7. Representative image of a 13-week-old graft are shown. A: PDX-1 (reddish brown) was demonstrated under the renal capsule in the grafted kidney at 40X magnification. B: Negative control with secondary antibody only. G, human fetal ICCs graft; K, murine kidney.

3.5.3 Infiltration of CD45 into transplanted ICCs grafts of 1st and 2nd trimester

Leukocytes infiltration to grafted kidney 1 week after transplantation was assessed by immunostaining of CD45. Extensive infiltration of mouse $CD45^+$ leukocytes was noted in kidney transplanted with second-trimester ICCs (Figure 3.31B and D). In contrast, a minimal infiltration occurred in kidney transplanted with first-trimester ICCs (Figure 3.31A and C).

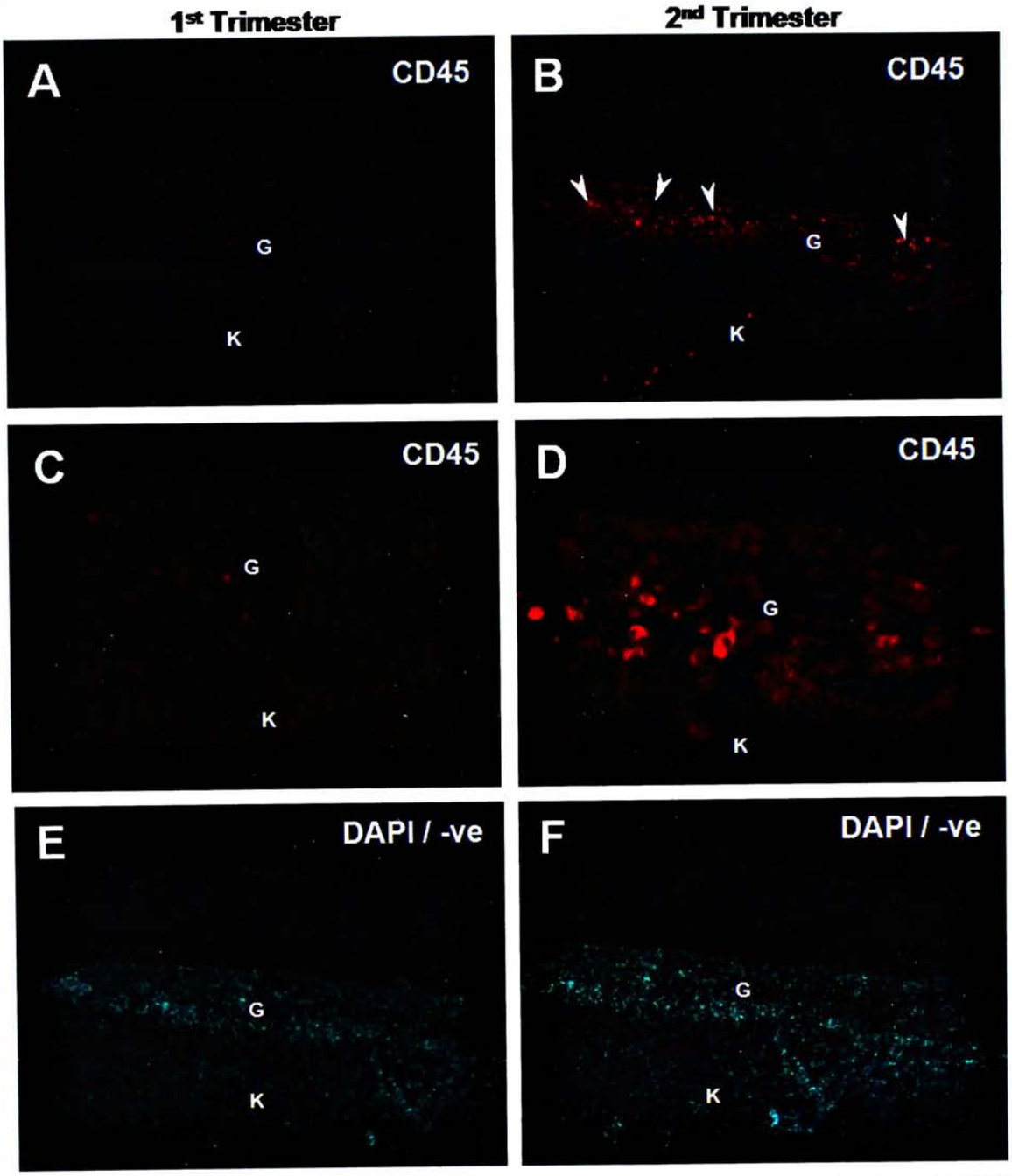

Figure 3.31 Immunostaining of CD45 in grafted kidneys of adult STZ-induced C57/BL6 diabetic mice having undergone ICC transplant for one week. Representative images of kidney transplanted with first and second trimester ICCs are shown. A-D: CD45 (red fluorescence) at 10X magnification (A-B) and 40X magnification (C-D). E-F: DAPI staining. G, human fetal ICCs graft; K, murine kidney.

CHAPTER 4

DISCUSSION

T1DM is an autoimmune disease resulting from the autoimmune destruction of pancreatic β-cells that leads to the lack of insulin production and failure in glucose homeostasis. Transplantation of either whole pancreas or islets is an alternative for traditional insulin administration. Among different sources of pancreatic tissues or pancreatic islets for transplantation, fetal pancreata have been proven as a promising approach for the potential for transplantation of functional β-cells in treating or even curing T1DM. This notion is further consolidated by the greater proliferative capacity of fetal pancreas (Hayek & Beattie, 1997), compared with adult tissues as well as its immune privileged status. Previous studies of the transplantation of fetal pancreas into diabetic immunoincompetent rodents consistently showed that they proliferate and differentiate into mature beta cells after transplantation (Hayek & Beattie, 1997; Castaing et al., 2001); more importantly, it also demonstrated successful amelioration of hyperglycemic conditions observed in experimental diabetes animals (Castaing et al., 2001; Hullett et al, 1987; Tuch et al., 1988; Tuch & Monk, 1991).

Apart from the functionality of these tissues or cells, another major concern about transplantation is the susceptibility to immune rejection. A lower immunogenicity has been reported in embryonic tissues when compared to adult tissues (Medawar, 1953).

Various fetal tissues, such as fetal kidney (Foglia et al., 1986; Dekel et al., 1997), fetal intestine (Lopes et al., 2000), and fetal skin (Erdag et al., 2002) have been consistently suggested to be more immune privileged when compared to their adult counterparts. Apart from comparison with adult tissues, it was also noted that the immunological status of the fetal tissue itself indeed correlates with the gestation age of the fetus from which the tissues were harvested. Previous studies addressing the immunogenicity of fetal kidney (Dekel et al., 2003) demonstrated that first-trimester human fetal kidney was less immunogenic than adult and second-trimester ones. Indeed, elder kidney was subjected to rejection upon transplantation into humanized mice, while kidney obtained from 7 to 8 weeks of gestation showed no sign of rejection. It was also shown that earlier pig embryonic pancreatic tissue harvested on embryonic day 42 (E42) exhibited a remarkable reduced immunogenicity in a xenotransplantation model when compared to E56 or later embryonic tissues (Eventov-Friedman, 2006). A more recent research revealed that human fetal pancreas from first trimester had a significantly decreased immunogenicity (Brands et al., 2008); transplantation of these human fetal pancreas further revealed a limited cellular infiltration in first-trimester grafts, whereas second-trimester grafts were completely infiltrated and rejected. In regard to these findings, the immunogenicity of haman fetal PPCs and ICCs from first and second

trimester has yet to be elucidated.

4.1 Expression of selected immuno-related genes in PPCs and ICCs

In order to investigate the inherent immunogenic properties of PPCs and ICCs, the expression of several selected immune-related genes in PPCs and ICCs were identified by RT-PCR in the present study. In this context, immunogenicity of the fetal kidney (Dekel et al., 2003) and fetal pancreas (Brands et al., 2008) comparing to their elder counterparts have revealed significant changes in expression of a number of genes that have direct roles in immune response. These genes were categorized into HLA molecules, cytokines, chemokines and their receptors, apoptotic related molecules, adhesion molecules, molecules of innate immunity and other immunomodulators. MHC molecules are among the most potent antigens responsible for allograft rejection (Colvin, 1990). In the present study, we demonstrated the mRNA expression of both MHC-I and MHC-II at both PPCs and ICCs levels (Figure 3.1). Previous reports have shown that undifferentiated hESC (Drukker et al., 2002; Li et al. 2004; Grinnemo et al. 2006) and human MSCs (Majumdar, 2003) express only MHC class I but no MHC class II molecules, while expressions of both MHC-I and II molecules were detected on human NSCs (Fu et al., 2010). These data showed the differential MHC expression

on different types of stem cells and they might contribute to different immunogenicity. More importantly, our present data showed that the mRNA expression of MHC-I in PPCs is 3-4 folds lower than that of fetal pancreas tissue at the same stage of development (Figure 3.2). The lower expression of MHC-I in PPCs may be partly due to the lack of the potentially immunogenic exocrine tissues and other mesenchyme, which are digested and removed during the isolation process of PPCs. The finding provides some clues that PPCs possess much less immunogenicity than that of fetal pancreas, and that it may be a better candidate for the production of insulin-producing islet-like cell clusters for clinical transplantation.

Meanwhile, the expression of several immune related genes, which are closely associated with T cell response, including C3, CCL19, TNFSF10 and B7H4 (Figure 3.1) were also detected in PPCs and ICCs. C3 and CCL19 have been reported to be involved in recruiting and stimulating T cells (Ngo et al., 1998; Froster et al., 1999; Pratt et al., 2002). Indeed, it was shown that the absence of local synthesized C3 in the renal grafts could result in remarkable improved graft survival in a murine model, implicating the potential role of C3 in modulating acute T-cell mediated rejection (Pratt et al., 2002). TNFSF10 has been reported to have co-stimulatory activity with T

cells (Chou et al., 2001; Tsai et al., 2004). B7H4, which is the newest member of the B7 family, is a negative regulator of T cell responses (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003). In light of these findings, it is plausible to speculate that difference in the expression of these immune related genes contribute directly to the differential immunological status of tissues or cells from different gestational ages.

Similar to MSC (Nauta & Fibbe, 2007) and hESC (Li et al., 2004; Grinnemo et al., 2006), the expression of co-stimulatory molecules CD80 and CD86 were not detected in PPCs and ICCs. The lacking of these co-stimulatory molecules might lead to T cells anergy (i.e. fail to respond to their specific antigen) or at least, to some extent, the delay in the triggering of immune response by T cells. Of particular interest is the focus on the expression of the non-classical MHC class-I molecules, HLA-G, in both PPCs and ICCs. HLA-G molecules have been reported as an immunomodulatory molecule that is capable of inhibiting the cytolytic function and proliferation of NK cells and cytotoxic T lymphocytes, as well as the maturation and function of dendritic cells (Carosella et al., 2008). HLA-G has also been reported as one of the factors associated with the immune modulating properties of MSCs (Rizzo et al., 2008). The existence of HLA-G in PPCs and their derivatives ICCs, though of low level, is

therefore indicative of the potential immunomodulatory characteristic of these cells.

4.2 Effects of IFN-γ and IL-10 on expression of immuno-regulated genes in PPCs and ICCs

The cytokine IFN-γ, secreted by thymus-derived cells and NK cells under certain conditions of activation, plays a complex and central role in the resistance of mammalian hosts to pathogens. IFN- γ was originally regarded as an antiviral agent (Wheelock EF, 1965) but was later discovered because of its multiple roles in the regulation of several aspects of immune response such as stimulation of bactericidal activity in phagocytes, orchestration of leukocyte-endothelium interactions and stimulation of antigen presentation through class I and class II MHC molecules (Boehm et al., 1997). The induction of MHC expression by IFN-γ is important for host response to intracellular pathogens since it increases the potential for T cell recognition of foreign peptides and therefore promotes the induction of cell-mediated immunity. Indeed, the IFN- γ inducible components are not only confined to the MHC complex but also to genes responsible for the processing and loading of peptides (Kern et al., 1995; Boehm et al., 1997; Seliger et al., 2001) IFN-γ has been extensively reported to induce the expression of MHC molecules in various cell types such as MSCs (Chan et al., 2006; Stagg et al., 2006; Chan et al., 2008), NSCs (Odeberg et al.,

2005), hESCs (Drukker et al. 2002; Li et al. 2004; Grinnemo et al. 2006) and tumor cells (Zhao et al., 2007). In this study, it was noted that IFN-γ upregulated mRNA expression of MHC-I on PPCs in a dose- and time-dependent manner (Figure 3.3). Flow cytometry revealed an increase of MHC-I intensity on PPCs after IFN-γ challenge (Figure 3.4). The current result is in line with previous studies indicative of the potential of IFN-γ in inducing the capacity antigen presentation in PPCs.

Apart from the potent inductive effect on MHC molecules, we have also examined whether IFN-γ is capable of inducing other immune related genes, in particular B7H4 and HLA-G., in PPCs. Previous studies demonstrated a limited capacity of IFN-γ in inducing B7H4 on freshly isolated human peripheral blood mononuclear cells (T cells, B cells, monocytes and DC), where B7H4 is initially absent (Sica et al., 2003). In a study of renal tubular epithelial cells, the induction of B7H4 expression needs not only IFN-γ but also the presence of other proinflammatory factors including TNF-α, IL-10 and lipopolysaccharide (Chen et al., 2006). In addition, several studies by Kryczek et al. have highlighted the role of IL-10, instead of IFN-γ, in inducing the expression of B7H4 on human overian cancer carcinoma (Kryczek et al., 2006) and APCs (Kryczek

et al., 2006). In contrast to the previous studies, dose-dependent increase of B7H4 expression by IFN-γ in PPCs was demonstrated in this study (Figure 3.8). The finding may arouse re-examination of the effect of IFN-γ on B7H4 as well as the possibility of IFN-γ-induced B7H4 expression to be tissue specific. As a negative regulator of T cells, the induction of B7H4 expression under IFN-γ challenge indicates the immune privileged status of PPCs.

On the other hand, treatment of the macrophages cell lines and monocytes with IFN-γ increased the mRNA, cell surface and intracellular level of HLA-G in a dose-dependent manner (Yang et al., 1996). These convergent results prompt us to conclude that the HLA-G is tissue specific, in which trophoblast and activated macrophages are able to produce HLA-G, while the HLA-G gene in fibroblast cannot be transcribed even stimulated with IFN- γ (Yang et al., 1995; Yang et al. 1996). This is justifiable by the investigation on the transcriptional regulation of HLA-G genes suggestive of the divergence of regulatory sequences in the promoters of HLA-G gene, when compared with HLA-E and –F, rendering the unresponsiveness to NF-kB, IRF1 and CIITA mediated induction pathways by IFN- γ (Gobin, 2000). In this study, the effect of IFN-γ on the HLA-G protein expression in PPCs was examined by Western

blotting (Figure 3.7). Compared with JEG-3, results revealed that the protein translation of HLA-G was weak in PPCs, while the protein level remained unchanged in both JEG-3 and PPCs even stimulated with IFN- γ for 2 days. The Western blot analysis appears to be contradictory to that of the mRNA expression (Figure 3.20) in which a 2-fold increase were observed in mRNA HLA-G by IFN-γ in PPCs of first-trimester. As IFN-γ regulates MHC-I in a dose- and time-dependent manner (Figure 3.3), further investigations need to confirm whether IFN-γ treatment at a higher dosage and longer period could display any effect on the protein expression of HLA-G. On the contrary, the mRNA and protein expression of HLA-G by PPCs were induced by IL-10. Several lines of evidence have suggested the potent role of IL-10 on regulating HLA-G expression, particularly the trophoblast and monocytes (Moreau et al., 1999). Furthermore, an upregulation of HLA-G by IL-10 is observed in decidual stroma cells (Blanco et al., 2008) and cutaneous lymphomas (Urosevic & Dummer, 2003). More importantly, a recent immunologic study on MSCs proposed that the inhibitory effect on T cells and NK cells and the induction of Tregs are mediated through the production of soluble HLA-G5 in an IL-10-dependent manner (Selmani et al., 2008). In the study, it was revealed that the pre-treatment of a moderate concentration of IL-10 on both PPCs and ICCs resulted in the upregulation in mRNA

expression of HLA-G (Figure 3.5 and 3.6). Finally, the protein expression of HLA-G in PPCs being congruous to that of JEG-3, as demonstrated in this study, was noted to increase after IL-10 treatment (Figure 3.7).

Taken together, the exploration of potential upregulation of MHC molecules, B7H4 and HLA-G levels, by cytokines such as IFN-γ and IL-10, in PPCs can be translated into in vitro manipulation of the immunological status of PPCs. Thus the elaboration of this scenario may help devise a better transplantation protocol by means of pharmalogical intervention of cells prior to transplantation.

4.3 In vitro studies on immunogenicity of PPCs and ICCs from 1st and 2nd trimester

4.3.1 Immune-related genes expression

A study by Rebel et al. reported that, during the development of hematopoietic stem cells, the expression of HLA class I and class II molecules would increase from no expression at the yolk sac stage to high expression in adult life (Rebel et al., 1996), suggesting the development of immunogenic markers during the ontology of cell across gestational stage. The present study revealed that a temporal change in the gene expression of MHC-I in the progenitor cells increased across 9 to 14 week, where a significant expression of MHC-I was observed after 13th week. Gene expression of MHC-II also demonstrated a temporal expression across gestational weeks, with a 100-fold increase in expression at 12th week and this level kept plateau since then. Comparing to the gene expression level of the two classes of MHC molecules, MHC-II showed a more drastic increase than MHC-I in terms of fold difference. Despite the dramatic change in MHC-II gene expression, the level of MHC-II is indeed much lower than MHC-I. From this temporal gene expression study, a different pattern of increase in these two genes was noted, implying the distinct development progress of MHC-I and MHC-II during the ontology of progenitor.

The differential expression of several genes previously identified in PPCs and ICCs

was studied. Similar to PPCs (Figure 3.11A and B), the expression of MHC-I and MHC-II in ICCs were found to be increased in second trimester (Figure 3.13A and B).Two other pro-inflammatory molecules including TNFSF10 and C3 were significantly upregulated in second trimester in both PPCs and ICCs (Figure 3.11 and 3.13). As both C3 and TNFSF10 participate in the stimulation of T cells, the higher expression of these genes in second-trimester PPCs and ICCs therefore implies a more vigorous T cell stimulation by these cells upon transplantation. On the other hand, the gene expression of B7H4 and HLA-G was lower in the second trimester PPCs and ICCs. The higher expression of these immune-suppressing genes indicate that the immune status in progenitors of first-trimester is more privileged than that of second-trimester. These data suggest that the expression of these particular immune-suppressing markers, unlike the MHC molecules, may be reduced along the gestational development of the fetus.

4.3.2 IFN-γ activated gene expression

The immunogenicity of PPCs and ICCs was elucidated by comparing the degree of upregulation of certain immune-related genes upon IFN-γ challenge. It is assumed that tissues or cells would be exposed to inflammatory cytokines such as IFN-γ (Bradley et al., 2002), which could induce MHC molecule expression and antigen presentation to accelerate T cell stimulation as a protective mechanism against the foreign grafts during transplantation. Herein, results of the study demonstrated an increase in gene expression of MHC-I at both PPC and ICC levels after treatment with IFN-γ for 2 days (Figure 3.15 and 3.17). Comparing to different trimesters, it was noted that the induction in mRNA expression of MHC-I was more intense in second trimester than first trimester. The finding was attested by flow cytometry that an increase in surface protein expression of MHC-I from PPCs and ICCs was significantly higher in second trimester (Figure 3.16 and 3.18).

Apart from MHC-I, the effect of IFN-γ on the gene expression of MHC-II, HLA-G and B7H4 in PPCs of first and second trimester were also investigated. It was noted an increase of over ten thousand-folds in gene expression of MHC-II in PPCs after IFN-γ treatments, and a more drastic increase was found in second trimester where the

change was 200,000 folds (Figure 3.19). MHC-II has been reported to play a main role in transplantation rejection by interaction with $CD4^+$ T helper cells (Archbold et al., 2008). The drastic upregulation of MHC-II molecules in PPC hence implies the susceptibility of PPCs, particularly from second trimester, in being induced immunogenic upon IFN-γ challenge.

Besides, although no noticeable change was observed in protein expression of HLA-G by IFN-γ as discussed in previous section, a slight increase in the mRNA level of HLA-G was noted in first-trimester PPCs (Figure 3.20), while concordant to the protein level, there was no change in the second-trimester. Furthermore data showed an increase in mRNA expression of B7H4 in both trimester but not different significantly (Figure 3.21).

4.3.3 Mixed lymphocyte reaction

Upon examination of the difference in the expression of immune related genes, the immunological function and the capacity of allogenic recognition and rejection of PPCs were studied by MLR. MLR has been extensively employed in many studies to determine the immunogenicity of certain type of stem cells. Neural stem cells were shown to trigger low but appreciable allogenic lymphocyte proliferation upon co-culture (Ubiali et al., 2007). While MSCs have been consistently reported to be capable in suppressing allogenic lymphocyte proliferation, irrespective of the site they were collected. Demonstration for the immunosuppressive effect was first reported in bone marrow derived MSCs from human (Di Nicola et al., 2002; Le Blanc et al., 2003; Potian et al., 2003; Tse et al., 2003), baboon (Bartholomew et al., 2002) and murine (Djouad et al., 2003; Krampera et al., 2003), showing that MSCs can inhibit T lymphocyte activation and proliferation in vitro. Interestingly, previous studies have shown that MSC-like cells from other tissues such as adipose tissue (Puissant et al., 2005) and fetal lung tissue (Götherström et al., 2004) also inhibit lymphocyte proliferation and inflammatory cytokine production. Study of Oh et al. revealed the suppressive effect of umbilical cord blood-derived MSCs on the allogenic proliferation of responder lymphocytes (Oh et al, 2008). In the present study, isolated PBMCs were

co-cultured with γ-irradiated PPCs, and evidence of DNA synthesis of this one-way MLR indicated the stimulation of growth of the untreated PBMCs by PPCs. Given that cells from fetal pancreas trigger proliferation of lymphocytes in a previous study (Tuch et al., 1988), in vitro functional test was employed to analyze the capability of the first- and second- trimester PPCs to be recognized by PBMCs, which may lead to rejection upon transplantation. Data showed that PPCs triggered the alloreactive proliferation of PBMCs in a dose-dependent manner and a low concentration of PPCs ($5X10^2$ cell/well) failed to elicit PBMCs proliferation (Figure 3.22). These results are therefore supportive of the possibility that PPCs stimulate PBMCs proliferation in an intimate manner. While most studies are in agreement that soluble factors are involved, as the separation of MSCs and PBMCs by transwell, prevented the inhibition of proliferation (Di Nicola et al., 2002; Tse et al., 2003; Rasmusson et al., 2003). It was also suggested that TGF-β and hepatic growth factor (HGF) were mediators of suppression of T-cell proliferation (Di Nicola et al., 2002). The possibility that some soluble factors may be released from PPCs to trigger the proliferation of PBMCs cannot be ruled out. Nevertheless, this notion needs further confirmation by means of the MLR in a transwell system using different concentrations of PPCs.

MLR was performed to assess the extent of immunological responses of the PBMCs. Strong proliferative responses (>300% compared to control) were observed (Figure 3.23). Compared to the allogenic control, the stimulation provoked by PPCs is rather low, but it was still indicative of the potential to act as non-professional APCs that stimulate the immune system. Data show that PPCs from second trimester elicited a higher proliferation in PBMCs than that of first trimester. A possible explanation of this phenomenon rests on the higher MHC-II expression as previously demonstrated since pretreatment of IFN-γ in PPCs of both groups showed a significant increase in allogenic stimulation in PBMCs, consistent with a remarkable increase in the expression of MHC-II molecules in PPCs. It is well recognized that MLR is predominantly related to MHC-II (Bach & Amos, 1967). It is therefore indicative of the chief role of MHC-II molecules in PPC-induced PBMCs proliferation. In the study, a higher proliferation of PBMCs was elicited in the presence of PPCs from second trimester, either in basal or IFN-γ pretreated conditions, suggesting that progenitor cells isolated from fetus of later gestational ages may probably be more immunogenic. These observations are in agreement with the notion that second trimester fetal pancreas is more immunogenic than first trimester counterparts, as evidenced previously (Brands et al., 2008). In addition, it was noted that stimulated PBMC

proliferation by PPCs of first trimester was abolished by pretreatment with IL-10. While an increase in IL-10 production in MSC/monocyte culture was reported (Aggarwal & Pittenger, 2005; Jiang et al., 2005; Beyth et al., 2005), the neutralization of the IL-10 antibody can partially restore T cell proliferation, thus suggesting a suppressive role of IL-10 in MLR (Beyth et al., 2005). More recent studies have also suggested the involvement of HLA-G in the immunosuppressive effect of MSCs (Nasef et al., 2007; Selmani et al., 2008). In view of this fact, the diminished PPC-induced PBMC proliferation by IL-10 may be explained by the upregulation of HLA-G expression in the cells.

4.3.4 Cytokines production of PBMC in MLR

A significant difference in cytokine production in MLR of 57 renal allograft patient/donor pairs was reported (Cartwright et al., 2000). In that study, a strong correlation between mitogen-simulated IFN-γ secretion and the rejection of renal graft was proposed. It is therefore plausible to measure the cytokine secretion in MLR in order to reflect the in vivo alloreactivity. The analysis of IFN-γ level in the supernatant of MLR with PPCs of second trimester and PHA-stimulated PBMCs exhibited a significant increase in the production of IFN-γ (Figure 3.24). Results appeared to be contradictory to those obtained from the MSCs that were shown to reduce IFN-γ production by PHA-sitmulated PBMCs (Aggarwal & Pittenger, 2005; Oh et al., 2008). In the PBMCs, NK cells and $CD4^+$ T cells are classically known as the two main sources for IFN-γ (please refer to section 1.5.1.3). It was verified in co-cultures of MSCs with CD4+ cells or NK cells that revealed a significant suppression of IFN-γ in the culture supernatant by MSCs (Aggarwal & Pittenger, 2005). Hence the IFN-γ produced in the MLR was most likely from T cells and NK cells in the PBMCs, which were subjected to the regulation of PPCs.

On the other hand, the production of the anti-inflammatory cytokine IL-10 by

activated DCs and Th2 cells was upregulated in the presence of MSCs (Aggarwal & Pittenger, 2005; Jiang et al., 2005; Beyth et al., 2005). In the study PPCs from both first and second trimester suppressed IL-10 production in vitro. Despite the discrepancy, data suggest the suppression of IL-10 production by Th2 cells (Ueta et al., 2002). There were also statistics showing that the renal rejection was in close relationship with increased IL-10 production in MLR (Cartwright et al., 2000). In view of this, it needs to be elucidated whether the production of IL-10 by DCs and T cells in MLR has an implication on a reduced immunogenicity. Results of the study showed that first-trimester PPCs might not stimulate the production of pro-inflammatroy cytokine IFN-γ in co-cultures with PHA-stimulated PBMCs, and hence supported the hypothesis that first trimester PPCs possess a lower immunogenicity over second trimester PPCs.

4.4 *In vivo* Xenotransplantation of ICCs into diabetic mouse model

Due to the severe shortage of adult donor tissue available to transplantation, alternative sources of β–cells, such as human or porcine fetal pancreas and insulin-producing cells derived from stem cells for transplantation are indispensable. ESC is one of the prominent and promising cell sources for the generation of insulin-producing β-cells. Previous studies showed that the transplantation of ESC-derived endocrine precursors resulted in insulin-positive cells that could rescue STZ-induced hyperglycemia in murine model (Soria et al., 2000; Kroon et al., 2008). More recently, the great potential of induced pluripotent stem cells (iPS) as an alternative to ESCs has been explored because of their capacity to give rise to insulin-producing cells (Tateishi et al., 2008). Nevertheless, the problem of the formation of teratomas by both ESCs and iPS are yet to be solved. The advantage of using organ specific progenitor cells depends on their lower tumorigenicity and specific differentiation pathways that require minimal in vitro manipulations so to obtain fully functional β-cells, compared with those from ESCs and iPS. Fetal liver progenitor cells were shown to be induced to differentiate into insulin-producing cells and these cells reverse the hyperglycemic conditions in immunodeficient mice (Zalzman et al., 2003; Zalzman et al., 2005). In this regard, it is amenable to isolate, characterize and culture transplantable ICCs derived from human

fetal PPCs (Suen et al., 2008). The PPCs isolated from human fetal pancreas represent another promising source of β-cells in treating T1DM. A lower immunogenicity in PPCs in vitro and their derived ICCs from first trimester was noted. As the immunological status of transplanted ICCs was not yet understood, a pilot study was conducted to investigate the in vivo immunogenicity of ICCs.

It is generally accepted that fetal tissues are less immunogenic than their adult counterparts. Renal grafts from adult and second trimester were noted to be completely rejected, whereas early kidney precursors showed no signs of rejection (Dekel et al., 2003). The study of human fetal pancreas demonstrated that tissue from first-trimester was less immunogenic than second-trimester grafts; extensive infiltration of mononuclear cells were observed when transplanted into humanized mice (Brands et al., 2008). Similar results were also found in xenotransplantation of fetal pig pancreas into humanized mice that tissues from embryonic day 42 had a remarkably reduced immunogenicity in terms of delayed rejection as well as superior glycemia normalization (Eventov-Friedman et al., 2006). In the current study, ICCs were transplanted into the kidney capsule of STZ-induced diabetic C57BL/6 mice. Histological examination of grafted kidney harvested from 2, 4, 7, 12 days

post-transplant revealed a localization of ICCs the cortex of kidney one week post-transplant (Figure 3.28). However, the size of observable xenografts was reduced drastically on day 12 post-transplant (Figure 3.28), implying that ICC xenografts were subjected to immune attack in C57BL/6 mice and rejected thereafter. Although xenogeneic islet can trigger both the humoral and cellular immune response of recipient, cellular immune response is believed to be more critical in xenograft rejection (Gill et al., 1989; Mirenda et al., 1998; Oberholzer et al., 1999). Previous findings have shown that mice treated with an anti-CD4 antibody did not reject xenogenic skin (Pierson et al., 1989) or islet grafts (Simeonovic et al., 1990). On the other hand, the depletion of $CD8^+$T cell did not have any remarkable improvement in the survival of these grafts. Accordingly, $CD4^+$T cells were proposed to be the predominant cells mediating the xenogenic immune response. Cytokines such as IL-1 and TNF are known to impair β-cell function in vitro and ultimately lead to cell death (Sandler et al., 1990). It was proposed that IL-1 released from the activated macrophages would pose an adverse effect on islet grafts which also contributed to the destruction of the xenogenic grafts (Koragren & Jansson, 1994). Immunohistological examination has also revealed the expression of mature β-cell marker PDX-1 localized on the grafted ICCs (Figure 3.29). Another study also showed the in vivo proliferation

and differentiation of human fetal pancreas into mass beta cells after transplanted into NOD/scid mice (Castaing et al., 2001). In contrast to these previous findings, the low expression of PDX-1 in the ICCs grafts might reflect the maturation arrest of the ICCs in vivo. This can be explained by the xenogenic immune responses that reject the ICCs.

In the present study, both ICCs from first and second trimester were not able to normalize blood glucose levels in diabetic mice. An initial drop of blood glucose was detected 2 days after the surgery, and the blood glucose gradually decreased from day 3 whereby the blood glucose of first-trimester grafts displayed more drastic decline than that of second trimester. A similar glucose profile, i.e drastic glucose levels below 11mM on day 1 and day 2 post-transplant followed by a gradual rebound of glucose was observed in a study where islets of different species (human, pig, SD rat and Lewis rat) were transplanted as xenografts into STZ-induced C57BL/6 mice (Triponez et al., 2000). It might account, in part, to the diffusion of graft-secreted insulin into surrounding circulation that provides a short-term remedy for hyperglycemia in the host. However, the possibility of the surgical procedure that might affect the host's blood glucose levels cannot be ruled out. In any case, the mechanism(s) require

intensive investigations. On the other hand, the failure of ICCs to normalize hyperglycemia in diabetic mice can be explained by the incomplete differentiation of ICCs into fully mature and functional islets which are able to produce sufficient insulin so as to reverse hyperglycemia. Alternatively, the xenogenic environment might not render the ICCs to full development in the kidney graft, and not to mention the rejection of the graft. Although the current protocol failed to reverse blood glucose levels, hyperglycemia in the mice transplanted with both first- and second- trimester grafts was sustained since day 5, and generally the blood glucose levels in first-trimester grafts were slightly lower than that of second trimester (Figure 3.26). These indicate a less extensive xenogenic rejection in ICCs derived from first trimester and a limited capacity in lowering blood glucose in the diabetic mice than mice transplanted with second trimester ICCs.

The lymphocyte infiltration into the ICCs grafts were examined by immunohistochemistry of Leukocyte common antigen (LCA), which is also called CD45. In this study, the amount of $CD45^+$ cells in the graft area of first-trimester ICCs was significantly lower than that in second-trimester grafts (Figure 3.30). Histology revealed an extensive infiltration of $CD45^+$ cells to ICCs in second trimester than that of first trimester, thus a more rapid rejection, consistent with the observation of higher

blood glucose levels in transplanted mice. The in vivo data are also in line with the in vitro results that second trimester was more immunogenic than first trimester in terms of basal and cytokine-induced immune-gene expression and a higher potency in inducing PBMCs proliferation in MLR.

4.5 Conclusion

In conclusion, the current study provides evidence for a reduced immunogenicity of human fetal PPCs from first trimester over second trimester. PPCs from first trimester express a significantly lower level of pro-inflammatory markers and a higher level of immune-suppressing markers than those of second trimester PPCs, even under the challenge of IFN-γ. First-trimester PPCs also possess a reduced alloreactivilty in MLR with PBMCs, as indicated by a reduced PBMCs proliferation and pro-inflammatory cytokine production. Transplantation of PPC-derived ICCs from first trimester showed a remarkably reduced cellular infiltration than that of second-trimester grafts. Taken together, human fetal PPCs from first trimester might be immunologically superior for islet transplantation in treating T1DM.

4.6 Further studies

While the present study has been focusing on the immunogenicity of human fetal PPCs, the immunological properties of ICCs would require complete illustration. Questions remain on whether there would be any alternation in the immunogenic makeup of ICCs, disregarding of their gestation age, during the differentiation process from PPCs. A more realistic animal model, such as humanized NOD/scid mice, should be employed for transplant in order to elucidate the alloreactivity in ICCs of different stage of maturation. Focus would also be on in-depth investigation on the potential immunomodulatory effect of IFN-γ and IL-10 on PPCs and ICCs for in vitro or in vivo therapeutic manipulation of the immunogenicity of these cells.

CHAPTER 5

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